Univ. of
Toronto
Library

Digitized by the Internet Archive
in 2010 with funding from
University of Toronto

http://www.archive.org/details/studiesreprints32rock

Med
RF

STUDIES

FROM

THE ROCKEFELLER INSTITUTE

FOR MEDICAL RESEARCH

UNIVERSITY OF TORONTO LIBRARY

REPRINTS

VOLUME XXXII

157774
8/12/20

NEW YORK

THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

1920

Results of the investigations conducted at The Rockefeller Institute, or elsewhere under its grants, are first reported in a variety of journals and publications. The reports are then assembled in volumes designated Studies from The Rockefeller Institute for Medical Research, of which this is Number XXXII. The Studies appear serially but at irregular intervals. The text of the original publications is in all respects followed in the Studies. The name, date, number, and pages of the journal in which each article originally appeared are printed above the title. To insure uniformity and simplicity of reference, plates and illustrations repeat the numbers used in the place of first publication.

R
111
R7
v.32

COPYRIGHT, 1920, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

CONTENTS.

PATHOLOGY AND BACTERIOLOGY.

NOGUCHI, HIDEYO. Influence of temperature upon the velocity of the complement fixation reaction in syphilis 1

GATES, FREDERICK L. Restorative effect of salts of magnesium and calcium after lethal doses of sodium oxalate..... 9

KLIGLER, I. J. Modifications of culture media used in the isolation and differentiation of typhoid, dysentery, and allied bacilli..... 17

AMOSS, HAROLD L., and EBERSON, FREDERICK. An experimental test of Nuzum's antipoliomyelitic serum..... 21

MARSH, PENELOPE. Survival of typhoid bacilli in sour milk.... 31

UHLENHUTH, EDUARD. The antagonism between thymus and parathyroid glands..... 37

UHLENHUTH, EDUARD. Further proof of the existence of a specific tetany-producing substance in the thymus gland..... 47

KLIGLER, I. J., and DEFANDORF, J. The endo medium for the isolation of *Bacillus dysenteriæ* and a double sugar medium for the differentiation of *Bacillus dysenteriæ*, Shiga and Flexner 51

KLIGLER, I. J. Note on the cross-agglutination of *Bacillus coli communis* and *Bacillus dysenteriæ* Shiga..... 55

WOLLSTEIN, MARTHA. A further study of experimental parotitis 59

AMOSS, HAROLD L., and HASELBAUER, PETER. The rat and poliomyelitis: an experimental study..... 69

GATES, FREDERICK L. A report on antimeningitis vaccination and observations on agglutinins in the blood of chronic meningococcus carriers..... 75

MACNIDER, WILLIAM DEB. The stability of the acid-base equilibrium of the blood in naturally nephropathic animals and the effect on renal function of changes in this equilibrium. I. A study of the acid-base equilibrium of the blood in naturally nephropathic animals and of the functional capacity of the kidney in such animals following an anesthetic..... 101

MacNider, William deB. The stability of the acid-base equilibrium of the blood in naturally nephropathic animals and the effect on renal function of changes in this equilibrium. II. A study of the efficiency of an alkali to protect the naturally nephropathic kidney against the toxic effect of an anesthetic 117

Wollstein, Martha, and Meltzer, S. J. Experimental chemical pneumonia 129

Wollstein, Martha, and Meltzer, S. J. Effect of intrabronchial insufflation of solutions of some inorganic salts 133

Noguchi, Hideyo, and Kaliski, David J. The spirochetal flora of the normal female genitalia 141

Noguchi, Hideyo. A comparative study of experimental prophylactic inoculation against *Leptospira icterohæmorrhagiæ* 143

Rous, Peyton, and Oliver, Jean. Experimental hemochromatosis 153

Rous, Peyton. Urinary siderosis. Hemosiderin granules in the urine as an aid in the diagnosis of pernicious anemia, hemochromatosis, and other diseases causing siderosis of the kidney 169

Cullen, Glenn E., and Taylor, Herbert D. Relative irritant properties of the chlorine group of antiseptics 183

Amoss, Harold L., and Marsh, Penelope. Standardization of antimeningococcic serum 203

PHYSIOLOGY AND PHARMACOLOGY.

Githens, T. S., Kleiner, I. S., Meyer, A. L., and Meltzer, S. J. A method of producing experimental shock 215

Githens, T. S., Kleiner, I. S., Meyer, A. L., and Meltzer, S. J. Influence of mere opening the abdomen, of state of shock, and of subsequent section of sciatic nerve upon the blood flow from the femoral vein in cats 217

Meltzer, S. J. The application of a concentrated solution of magnesium sulfate to scalds and burns 219

CHEMISTRY.

LEVENE, P. A., and TAYLOR, F. A. Reduction of aldehydes to corresponding alcohols. I. Reduction of heptylic aldehyde (oenanthol) 223
LEVENE, P. A., and WEST, C. J. Cephalin. V. Hydrocephalin of the egg yolk 227
LEVENE, P. A. Epimeric hexosaminic acids 233
LEVENE, P. A. The action of nitrous acid on epimeric hexosaminic acids 249
LEVENE, P. A., and LÓPEZ-SUÁREZ, J. Mucins and mucoids ... 255
JACOBS, WALTER A., HEIDELBERGER, MICHAEL, and ROLF, IDA P. On nitro- and aminoaryl arsonic acids 277

EXPERIMENTAL BIOLOGY.

LOEB, JACQUES. The influence of electrolytes upon the osmotic pressure of gelatin solutions 291
LOEB, JACQUES. Amphoteric colloids. I. Chemical influence of the hydrogen ion concentration 303
LOEB, JACQUES. The law controlling the quantity of regeneration in the stem of *Bryophyllum calycinum* 325
LOEB, JACQUES. Amphoteric colloids. II. Volumetric analysis of ion-protein compounds; the significance of the isoelectric point for the purification of amphoteric colloids 341

THE HOSPITAL OF THE ROCKEFELLER INSTITUTE.

DERNBY, KARL GUSTAV. A study on autolysis of animal tissues. 359
CULLEN, GLENN E., and CHESNEY, ALAN M. A note on the production of acid by pneumococci 401
DERNBY, KARL GUSTAV, and AVERY, O. T. The optimum hydrogen ion concentration for the growth of pneumococcus .. 409
DOUST, BREWSTER C., and LYON, ARTHUR BATES. Face masks in infections of the respiratory tract 423
VAN SLYKE, DONALD D. Studies of acidosis. XI. The determination of carbon dioxide in carbonates 433
GOTO, KINGO. Studies of acidosis. XII. Mineral metabolism in experimental acidosis 437

ANIMAL PATHOLOGY.

JONES, F. S. Studies in bovine mastitis. II. The relation of hemolytic streptococci to udder infections 459
SMITH, THEOBALD. A pleomorphic bacillus from pneumonic lungs of calves simulating actinomyces 475
SMILLIE, ERNEST W. An improvement in the method of isolating and recovering the bacillus of cattle abortion through guinea pigs 487
BROWN, JAMES HOWARD. A new type of syringe, especially adapted for intravenous injections or the aspiration of blood 509
BROWN, JAMES HOWARD, and ORCUTT, MARION L. A rapid differential method for the isolation of *Bacillus influenzæ* 515
SMITH, THEOBALD. Spirilla associated with disease of the fetal membranes in cattle (infectious abortion) 519
JONES, F. S. Studies in bovine mastitis. III. Infection of the udder with micrococci and other microorganisms 539
JONES, F. S. Studies in bovine mastitis. IV. The sources of infection in streptococcic mastitis 553
TENBROECK, CARL. The survival of the hog-cholera virus in laboratory animals, particularly the rat 567
TENBROECK, CARL. A study of paratyphoid bacilli isolated from cases of hog-cholera 577

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1918,
Vol. xxviii, No. 3, pp. 297–303.]

INFLUENCE OF TEMPERATURE UPON THE VELOCITY OF THE COMPLEMENT FIXATION REACTION IN SYPHILIS.

BY HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 25, 1918.)

It has become customary to carry out immunity reactions *in vitro* at the temperature of 37°C., because most of the reactions between specific antibodies and antigens take place only at 37°C. or near that point. The reactions of bacteriolysis, cytolysis, hemolysis, agglutination, precipitation, phagocytosis, opsonization, etc., manifest their maximum activities at 37°C. Certain biological reactions, however, do not necessarily follow this general rule. For example, hemolysis produced by saponin, bile salts, cobra lecithid, or sodium oleate is complete within a very short time whether at 4° or 37°C.[1, 2] The velocity of the reaction in these instances is such that time and temperature play but a slight part.

The mechanism of the lipotropic complement fixation of syphilitic serum or spinal fluid is not understood, but it is certain that the lipoids are an important factor. The question in regard to the velocity of this reaction has not received as much attention as it deserves. The prevailing idea is that it is one of the immunity reactions which manifest their maximum activity at 37°C. Whether or not the reaction can take place at lower temperatures has not been carefully studied. We have been interested in this phase of the problem and have carried out numerous experiments to determine the relation between time, temperature, and reaction. The determination

[1] Madsen, T., and Walbum, L., Toxines et antitoxines. L'influence de la température sur la vitesse de réaction. I, *Overs. k. Danske Vidensk. Selsk. Forh.*, 1904, No. 6, 425.

[2] Madsen, T., and Noguchi, H., Toxines et antitoxines. L'influence de la température sur la vitesse de réaction. II, *Overs. k. Danske Vidensk. Selsk. Fork.*, 1904, No. 6, 447.

of these points is of practical importance at the present time, since, should the reaction prove to take place satisfactorily at a temperature which can be obtained without the aid of a special incubator, the performance of the test becomes much more widely adaptable. That this is the case is shown in the following experiments.

Detection of Varying Known Quantities of the Fixing Substance ("Syphilitic Antibody") at Different Temperatures.

It was the purpose in this series of experiments to study whether or not a given quantity of the fixing substance can be detected, not only at the usual incubation temperature of 37°C., but also at 30° or 23°. Graduated doses of a strongly positive syphilitic serum were chosen, so that a series would represent two and one fixing units and three-fifths and one-fifth of a unit. For the first experiment a serum was selected which produced complete fixation in a dose of 0.05 cc. within 30 minutes at 37°C. (water bath). Of this 0.1, 0.05, 0.03, and 0.01 cc. were measured into different tubes and tested for their fixing capacity at different temperatures, 37°, 30°, and 23°C.

In the first series human complement, 0.1 cc., and in the second guinea pig complement, 0.04 cc. (or 0.1 cc. of 40 per cent guinea pig serum dilution), were used. The quantities of the other elements were as usual, antihuman amboceptor, 0.1 cc., containing one unit for the human and two units for the guinea pig complement, 10 per cent human corpuscle suspension, 0.1 cc., and antigen, 0.1 cc. The total volume in each tube was made 1.5 cc.

Four groups of tubes, representing 0.1, 0.05, 0.03, and 0.01 cc. of the serum, were prepared for studying the influence of temperature upon the velocity of the reaction (Table I). Each group had seven duplicate sets for each dose of serum, as it was necessary to add amboceptor and corpuscle suspension at seven different intervals after the first incubation period began. The addition of the amboceptor and corpuscle suspension was made simultaneously 10 minutes, 20 minutes, 30 minutes, 60 minutes, 2 hours, and 4 hours after the contents were mixed.

For the temperature of 37°C. a water bath was used, for 30°C. a special thermostat room, and for 23°C. the laboratory room. Not

only the first incubation, but also the second or hemolytic phase of the reaction, was carried out at the temperatures indicated; that is, the results recorded under the heading of 23°C. were obtained at that temperature throughout, and those at 30° and 37°C. also.

In the experiments recorded in Tables I and II the same quantities of fixing substance ("syphilitic antibody") were employed, but the source and amount of complement used were different. It will be noted that the reaction was somewhat stronger when 0.04 cc. of guinea

TABLE I.

Fixation of Human Complement at Various Temperatures.

Incubation period.	37°C.				30°C.				23°C.			
	Serum.											
	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.
Immediately.	−	−	−	−	−	−	−	−	−	−	−	−
10 min.	++++	++	−	−	+++	−	−	−	+++	−	−	−
20 "	++++	+++	+	−	++++	++	−	−	++++	+	−	−
30 "		++++	++	±	++++	+++	+	−	++++	++	±	−
60 "		++++	++	±		++++	++	±	++++	+++	+	−
2 hrs.						++++	++	±	++++	++++	++	±
4 "						++++	++	±	++++	++++	++	±

Human complement, 0.1 cc.; diluted antihuman amboceptor, 0.1 cc. (one unit); 10 per cent human corpuscle suspension, 0.1 cc.; antigen, 0.1 cc. Total volume, 1.5 cc.

The second incubation also was carried out at the temperatures indicated.

pig complement was used instead of 0.1 cc. of human complement. The difference seems to be due to the fact[3] that the more active the complement the easier it is fixed, since guinea pig serum contains the requisite complement in a much smaller volume.

Some striking facts are brought out in these experiments. When the amount of fixing substance exceeds two units (0.1 cc.), complete

[3] Noguchi, H., and Bronfenbrenner, J., The interference of inactive serum and egg-white in the phenomenon of complement fixation, *J. Exp. Med.*, 1911, xiii, 92.

fixation takes place within 20 minutes at 23° or 30°C., while at 37°C. the fixation is complete within 10 minutes. When one unit of fixing substance (0.05 cc.) is used, the fixation is complete within 30 minutes at 37°C., 60 minutes at 30°C., and 2 hours at 23°C. With three-fifths (0.03 cc.) and one-fifth (0.01 cc.) of the fixing unit the minimum time required for completion of the fixation is 30 minutes for 37°C., 60 minutes for 30°C., and 2 hours for 23°C.

TABLE II.

Fixation of Guinea Pig Complement at Various Temperatures.

Incubation period.	37°C.				30°C.				23°C.			
	Serum.											
	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.	0.1 cc.	0.05 cc.	0.03 cc.	0.01. cc.
Immediately.	−	−	−	−	−	−	−	−	−	−	−	−
10 min.	++++	+++	+	−	++++	+++	−	−	+++	++	−	−
20 "	++++	++++	++	−	++++	+++	+	−	++++	+++	±	−
30 "	++++	++++	++	±	++++	++++	++	−	++++	+++	+	−
60 "	++++	++++	++	±	++++	++++	++	±	++++	++++	++	−
2 hrs.					++++	++++	++	±	++++	++++	++	±
4 "					++++	++++	++	±	++++	++++	++	±

40 per cent guinea pig complement, 0.1 cc.; diluted antihuman amboceptor, 0.1 cc. (representing two units for this system); 10 per cent human corpuscle suspension, 0.1 cc.; antigen, 0.1 cc. Total volume, 1.5 cc.

The second incubation also was carried out at the temperatures indicated.

The velocity of the reaction of complement fixation and subsequent hemolysis is much greater at 37°C. than at 30° or 23°C. A quantity of complement which will completely hemolyze the mixture in 20 minutes at 37°C. will require 35 minutes at 30°C. and 45 minutes at 23°C. for complete hemolysis. It is therefore understood that the test of syphilitic serum at 30° or 23°C. requires a period of nearly 3 hours, while at 37°C. the reaction is complete at the end of 1 hour.

TABLE III.

Comparative Study of Specimens Tested at Different Temperatures.

Specimen.	Amount.	37°C. Incubation 30 min.	30°C. Incubation 2 hrs.	23°C. Incubation 2 hrs.
	cc.			
Serum 1	0.2	−	−	−
" 2	0.2	++++	++++	++++
" 3	0.2	−	−	−
" 4	0.2	−	−	−
" 5	0.2	−	−	−
" 6	0.2	++++	++++	++++
" 7	0.2	++++	++++	++++
" 8	0.2	−	−	−
" 9	0.2	+++	+++	+++
" 10	0.2	−	−	−
" 11	0.2	++	++	++
" 12	0.2	−	−	−
" 13	0.2	++++	++++	++++
" 14	0.2	+	+	+
" 15	0.2	−	−	−
" 16	0.2	−	−	−
" 17	0.2	−	−	−
" 18	0.2	±	±	±
" 19	0.2	−	−	−
" 20	0.2	−	−	−
Cerebrospinal fluid 1	0.5	++++	++++	++++
Cerebrospinal fluid 2	0.5	++++	++++	++++
Cerebrospinal fluid 3	0.5	−	−	−
Cerebrospinal fluid 4	0.5	−	−	−
Cerebrospinal fluid 5	0.5	+++	+++	+++

Human complement was employed. Diluted antihuman amboceptor, 0.1 cc. (one unit); 10 per cent human corpuscle suspension, 0.1 cc.; antigen, 0.1 cc. Total volume, 1.5 cc.

The first and second incubations were carried out at the temperatures indicated.

Examination of Specimens of Serum and Cerebrospinal Fluid at Different Temperatures.

With a view to the possible practical application of the facts just mentioned, a series of tests was undertaken with specimens of serum and cerobrospinal fluid at 37°, 30°, and 23°C. The serum was used in the active state, and the cerebrospinal fluids were tested by adding human complement, 0.1 cc., to each specimen. Table III gives the results.

There was no disagreement in the results obtained at the three different temperatures. Apparently the examination of syphilitic sera and cerebrospinal fluids can be conducted in a warm laboratory without recourse to an incubator in which a temperature of 37°C. is maintained. It is advisable, however, to make use of the incubator whenever it is available, and, when not accessible, to make the room as warm as possible, in order to accelerate the reaction. 1 hour of incubation is sufficient when the temperature of the air is 32–37°C.

CONCLUSIONS.

Examination of syphilitic serum or cerebrospinal fluid can be made at any temperature between 23° and 37°C. The velocity of the fixation reaction, including the fixation of complement and subsequent hemolysis, is greater at a higher temperature, the optimum point being 37°C. The maximum reaction is also reached, however, when the mixture of lipoids, syphilitic serum, and complement is allowed to stand for a long enough period at a lower temperature, the minimum thermal point being near 23°C. For the optimum temperature (37°C.) an incubation of 30 minutes is sufficient, while for the minimum temperature (23°C.) 2 hours are necessary. At the temperature of 30°C. the reaction proceeds with moderate velocity and is complete within 60 minutes.

Guinea pig complement gave a sharper reaction with the sera which contained less than one unit of the fixing substance. Fixation is complete, however, at any of the three temperatures within 20 minutes when there are more than two units present. A serum containing one unit of fixing substance will complete reaction within 30 minutes at

37°C., 60 minutes at 30°C., and 2 hours at 23°C., irrespective of whether human or guinea pig complement is used.

For many reasons a properly adjusted thermostat for 37°C. is recommended for conducting the serum diagnosis of syphilis when possible, but it should not be overlooked that at a temperature near 30°C. an entirely reliable result can be obtained without a special incubator. Even at a temperature as low as 23°C. the test can be carried out if sufficient length of time is allowed.

The foregoing conclusions refer only to the systems in which the acetone-insoluble fraction of tissue lipoids[4] is used as antigen.

[4] Noguchi, H., Serum diagnosis of syphilis and butyric acid test for syphilis, Philadelphia, 3rd edition, 1912.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1918, Vol. xxviii, No. 3, pp. 305–312.]

RESTORATIVE EFFECT OF SALTS OF MAGNESIUM AND CALCIUM AFTER LETHAL DOSES OF SODIUM OXALATE.

BY FREDERICK L. GATES, M.D.

(*From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 16, 1918.)

In a recent article[1] Gates and Meltzer reported a series of experiments on rabbits in which the combined action of sodium oxalate and magnesium sulfate and the antagonistic action of calcium chloride on the effects of injections of these salts were employed. The experiments showed that while the symptoms resulting from intramuscular and subcutaneous injections of sodium oxalate differ markedly from the depressant effects of magnesium salts, a combination of subminimal doses of the two substances in the same animal causes a profound depression essentially similar to that which follows an effective dose of magnesium alone, the additional injection of the oxalate seeming to augment the magnesium effect. The anesthesia and paralysis resulting from the double injection differ, however, from those produced by magnesium alone by the presence of a plateau at the period of deepest depression, so that the condition of the animal remains apparently unchanged for a considerable period of time, during which respiration is fairly well supported. The antagonistic action of calcium salts proved to be as effective and striking as in the depression caused by magnesium alone.

In the present paper a series of experiments is reported in which the antagonistic action of salts of magnesium and of calcium was studied in rabbits which had received a lethal dose of sodium oxalate. Since the symptoms produced by magnesium are of a character opposite to those of acute oxalate poisoning, and since calcium is a precipitant of the oxalates in the animal body, as well as *in vitro*, it seemed

[1] Gates, F. L., and Meltzer, S. J., *J. Exp. Med.*, 1916, xxiii, 655.

possible that either magnesium or calcium might be of value in combating the effects of large doses of oxalate.[2]

Rabbits were employed. Sodium oxalate[3] in 3 per cent solution, and hydrated magnesium sulfate in molecular solution were injected into the sacrospinal muscles on opposite sides of the body. The doses were computed in grams per kilo of body weight. The site of the magnesium injection was always massaged for about 15 seconds, that of the oxalate was only sometimes massaged. Calcium chloride in $\frac{M}{8}$ solution was injected into the marginal vein of the ear in doses of 5 or 10 cc.

Effects of Sodium Oxalate.

When a lethal dose of sodium oxalate is injected into the lumbar muscles of a rabbit, a definite and characteristic train of symptoms follows. Often the animal defecates immediately after the injection. In the course of a few minutes it becomes restless and alert, changing position and posture suddenly, with quick abrupt movements. The hind legs seem to be stiff and are dragged. The respiration increases in depth and rapidity, and becomes somewhat labored, or panting, very rapid and continuous, without pause. The body shakes and the head nods with the breathing. A favorite posture is prone on the belly, with fore and hind legs extended, head up, and ears erect. The animal frequently gets up from this position only to seek it again after a moment of restless movement. After 10 or 12 minutes more acute symptoms make their appearance. Gradually the nodding head is retracted back and upward until the nose is in the air. At intervals it is jerked down again, only to rise as before. The respiration becomes more labored and irregular, and stops as the rabbit suddenly goes into a tetanic state with marked opisthotonos, followed by a violent spastic convulsion, and then becomes limp. Sometimes this first convulsion is terminal; the animal relaxes, and all movement ceases after a fading, final series of gasps during which the heart cannot be felt. In other instances the heart continues to beat, respiration returns, and a second period of restlessness may be followed at

[2] See also Starkenstein, E., *Zentr. Physiol.*, 1914, xxviii, 63; *Arch. exp. Path. u. Pharmakol.*, 1914, lxxvii, 45.

[3] Merck's reagent, Sörensen's oxalate.

intervals by another spastic struggle or a series of them. The successive periods between convulsions are marked by progressive weakness. The animal sprawls on the floor with legs spread, flanks relaxed, and head fallen to one side. Respiration becomes shallower, but not much slowed in rate. On palpation the heart beats seem to be rapid and weak. Finally the animal fails to recover from one of the convulsions, respiration fades away, the pupil dilates widely, and the heart can no longer be felt. A series of experiments with large doses of sodium oxalate is summarized in Table I.

TABLE I.

Determination of the Fatal Dose of Sodium Oxalate for Rabbits.

Sodium oxalate, 3 per cent solution; left lumbar muscles.

Dose per kilo.	Massage.	No. of experiments.	Lived.	Died.	Mortality.
gm.					*per cent*
0.12	None.	3	3	0	0
0.15	"	5	4	1	20
0.16	"	3	1	2	66
0.18	"	2	0	2	100
0.20	"	6	0	6*	100
0.20	15 sec.	5	0	5	100

* One death at 2 hours, 11 min., after a feeble convulsive struggle.

Of eleven rabbits which received an injection of 0.2 gm. per kilo of sodium oxalate, ten died in from 14 to 31 minutes after injection. The surviving animal succumbed in coma 2 hours, 11 minutes later. 0.2 gm. of sodium oxalate, given intramuscularly even without massage, is shown to be a fatal dose for rabbits. This dose was therefore selected for use in the next series of experiments.

Restorative Effect of Magnesium Sulfate.

A large amount of evidence shows that the symptoms which follow injections of magnesium salts are of a character opposite to those described for sodium oxalate. Shortly after the injection of magnesium the respiration may increase in rate, but soon becomes slower than normal and the animal sinks quietly into a progressive state of

narcosis or depression, with partial or complete anesthesia, loss of the superficial reflexes, and a varying degree of paralysis which may cause death by involvement of the respiration. Symptoms of hypersensitiveness and excitation are not seen. Hence we endeavored to counteract the effects of oxalate poisoning by injections of magnesium sulfate.

Only comparatively small doses of magnesium could be used. The earlier experiments had shown the striking action of oxalate in augmenting the effects of magnesium injection. Doses of 0.7 gm. of magnesium sulfate and 0.12 gm. of sodium oxalate, which when given alone produce only trivial symptoms, killed by respiratory paralysis three out of four rabbits. The dose of magnesium to be sought, therefore, was one which would counteract the acute symptoms of

TABLE II.

Restorative Effect of Magnesium Sulfate.

Magnesium sulfate M solution; right lumbar muscles. Site massaged.
Sodium oxalate 3 per cent solution; dose 0.2 gm. per kilo; left lumbar muscles.

Magnesium sulfate per kilo.	Sodium oxalate injection.	No. of experiments.	Lived.	Died.	Mortality.
gm.					*per cent*
0.6	Massaged.	10	3	7	70
0.4	"	10	4	6	60
0.4	Not massaged.	10	8	2	20
0.3	" "	1	0	1	100

oxalate poisoning, and yet not endanger the respiration through the depressant action of the combined salts. The experiments of this series are summarized in Table II. The injection of the magnesium sulfate followed immediately the injection of the lethal dose of the oxalate.

Table II correlates the results of a series of thirty experiments in which a dose of 0.6 or 0.4 gm. of magnesium sulfate was given to rabbits immediately after a lethal dose (0.2 gm.) of sodium oxalate. Of the ten rabbits which received 0.6 gm. of magnesium intramuscularly with massage, the oxalate injection being followed by massage also, only seven died. Death in all instances was due to respiratory paralysis. This dose of magnesium is effective in preventing oxalate

convulsions, but is in itself to a considerable degree dangerous to life when combined with so much oxalate. In a series of ten animals which received a dose of 0.4 gm. of magnesium sulfate, with massage of the site of the oxalate injection, four survived, there was one instance of a typical acute oxalate death, while five other animals died at later periods from the magnesium-oxalate depression. When, however, the site of the oxalate injection was not massaged, so that absorption occurred more slowly, 0.4 gm. of magnesium sulfate did prevent oxalate convulsions and reduced the mortality by 80 per cent. Of the ten animals of this series the two that died did not succumb in an acute attack, but died later from respiratory paralysis. In one experiment 0.3 gm. of magnesium sulfate, given to a rabbit whose oxalate injection was not massaged, did not prevent early death.

The experiments show that magnesium, when administered in proper doses, is antagonistic to oxalate poisoning. It is capable of reducing the mortality and can effectively counteract the convulsive symptoms which are characteristic of large doses of sodium oxalate.

The rabbits in which the magnesium injection was effective in preventing oxalate convulsions, and which survived the early period of acute oxalate symptoms, exhibited a depression similar to, but apparently not identical with that produced by subminimal doses of magnesium and sodium oxalate as described in our previous paper. This depression appears to be due to a specific inhibition of the nervous system, both central and peripheral, characterized by deep narcosis, with paralysis, anesthesia, and loss of the superficial reflexes. The depression following large doses of oxalate, with subminimal doses of magnesium, does not lead to a safe narcosis, from which the animal may be readily aroused by calcium. The depression is rather a type of coma, an intoxication marked by progressive weakness, in which the power of voluntary movement, sensitiveness to stimuli, and partial reflex activity remain until shortly before death, though these responses are, of course, limited by the extreme general intoxication of the animal. Calcium rouses these rabbits to some extent. In these animals effects seem to have occurred which are not readily reversed by calcium, as is the case in magnesium depression; and even when roused they show weakness and stupor, and soon sink back into their former comatose state.

Restorative Effect of Calcium Chloride.

In another series of experiments intravenous injections of calcium chloride in $\frac{M}{8}$ solution were made. After the lethal dose (0.2 gm.) of sodium oxalate had been injected intramuscularly, with or without massage, the calcium solution in doses of 5 or 10 cc. was injected into the marginal vein of the ear, either immediately, or after an interval of 10 to 12 minutes. These later injections anticipated the onset of the first convulsions, which were due to appear about 13 minutes after the injection of the oxalate. The results are given in Table III.

TABLE III.

Restorative Effect of Calcium Chloride.

Sodium oxalate injection.	Calcium chloride.		No. of experiments.	Lived.	Died.
	Time.	Amount.			
	min.	*cc.*			
Massaged.	10–12	5	1	0	1
"	10–12	10	3	1	2
"	1	5	1	0	1
"	1	10	3	3	0
Not massaged.	10–12	5	1	1	0
" "	10–12	10	1	1	0
" "	1	5	3	3	0
" "	1	10	2	2	0

The experiments show that intravenous injections of calcium chloride may save life after lethal doses of sodium oxalate. If the site of the oxalate injection is massaged, at least 10 cc. of $\frac{M}{8}$ calcium chloride, given immediately, are required; if the site is not massaged, 5 cc., even when given after 10 minutes, may be effective.

DISCUSSION AND SUMMARY.

The experiments presented in this paper brought out the following facts. Intramuscular injections of sodium oxalate into rabbits in doses of 0.18 and 0.2 gm. proved to be invariably fatal, death generally occurring in a comparatively short time. The symptoms consisted in excitation and tonic and clonic convulsions of diminishing strength if death was delayed. Some animals succumbed in the first

convulsion. From the experiments in which magnesium or calcium was added, it was evident that massage of the site of the oxalate injection is unmistakably an aggravating factor.

In the experiments with the addition of magnesium or calcium, doses of 0.2 gm. of oxalate were used, with or without massage. The character of the effect which follows an addition of magnesium and calcium was studied in animals which received oxalate in doses above the minimal lethal one. Nevertheless, there was practically in all cases clear evidence that the effect of the additional injections was in the nature of an antagonism to the oxalate effects. An injection of 0.6 gm. of magnesium not only alleviated or abolished the excitation and convulsions, characteristic of oxalate poisoning, but also reduced the mortality by 30 per cent. When the dose of magnesium was only 0.4 gm. per kilo of body weight and the site of the injection of the oxalate was not massaged—a condition in which 0.2 gm. of oxalate alone was invariably fatal—the mortality was reduced by 80 per cent.

The favorable effect of injections of calcium chloride depended upon the quantity injected and the length of the interval elapsing between the injection of the oxalate and that of the calcium. When the site of the oxalate was massaged and only 5 cc. of the calcium solution were injected, the animals succumbed to the oxalate poisoning. When 10 cc. of calcium were given 1 minute after the oxalate injection, the animals survived. When the site of the oxalate injection was not massaged, then even 5 cc. of the calcium were sufficient to save life.

A curative effect of calcium upon oxalate poisoning has been claimed by previous writers. This is not difficult to explain and probably consists in the simple chemical process which can be demonstrated *in vitro*. Oxalates precipitate calcium salts *in vitro* and have the same effect within the animal body. By virtue of this precipitation the calcium of the body is reduced below the amount indispensable to maintain life. Loeb said: "It is due to the presence of Ca- (and K-) ions in our blood that our muscles do not contract rhythmically."[4] The convulsive movements in oxalate poisoning may be due at least

[4] Loeb, J., *Am. J. Physiol.*, 1899–1900, iii, 327. See also Meltzer, S. J., and Auer, J., *ibid.*, 1908, xxi, 400.

partly to a reduction of the calcium content of the fluids of the animal body. In our experiments it required an injection of 10 cc. of calcium chloride to restore the calcium content to the indispensable amounts; the injection of 5 cc. was effective only when the site of the oxalate injection was not massaged.

The fact that calcium salts are antagonistic to the effects of magnesium salts, which are practically only inhibitory in their nature, does not exclude the possibility that under certain conditions calcium may also exert an inhibitory effect. The following is an instructive illustration. Prolonged perfusion of a nerve muscle preparation with sodium chloride abolishes the irritability of the motor nerve endings; addition of calcium chloride restores it. On the other hand, perfusion with calcium chloride alone promptly abolishes this irritability. In one case it restores the irritability while in the other it inhibits it.[5]

It is different with regard to the action of magnesium upon oxalate poisoning. Superficially it seems that there is a direct contradiction between the results reported in a previous communication[1] and the experiments reported in this paper. The first series of experiments demonstrates that oxalate and magnesium act synergetically, while in the present series evidence is brought forward that magnesium acts antagonistically to the poisonous effects of the oxalate. However, this seeming contradiction may be explained on the assumption that oxalates, especially in larger doses, aside from their calcium-precipitating property, exert by means of a yet unknown factor a further toxic effect which favors the development of excitation and convulsions, which in turn lead to exhaustion and death. The employment of carefully selected doses of a magnesium salt will reduce or abolish the excitation and spastic attacks and thus will prevent exhaustion and hence save life. We may thus say that the favorable antagonistic action of magnesium against large, fatal doses of an oxalate is merely symptomatic in its nature—similar, for instance, to the favorable effect of chloroform upon strychnine, convulsions—while the synergetic action of subminimal doses of magnesium and oxalate may be considered as specific in nature. The precipitating action of the oxalate decreases the calcium content and thus increases the effectiveness of the inhibitory action of the subminimal dose of magnesium.

[5] Joseph, D. R., and Meltzer, S. J., *Am. J. Physiol.*, 1911–12, xxix, 1.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1918,
Vol. xxviii, No. 3, pp. 319–322.]

MODIFICATIONS OF CULTURE MEDIA USED IN THE ISOLATION AND DIFFERENTIATION OF TYPHOID, DYSENTERY, AND ALLIED BACILLI.*

BY I. J. KLIGLER, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 20, 1918.)

A comparative study of some of the media proposed for the isolation of the typhoid group of bacilli led to the conclusion that the Endo and brilliant green plates are most satisfactory. Both of these media, however, possessed certain defects which impaired their usefulness. It is believed that the modifications reported below tend to eliminate these defects.

Modification of the Endo Medium.

It was observed that the Endo medium, as ordinarily prepared, with a phenolphthalein reaction of + 0.2, gave variable results with the dysentery group of bacilli, the Shiga type of *Bacillus dysenteriæ* being either markedly inhibited in its growth or failing to grow at all. This restraining effect was found to be due to the end-reaction, which, after the addition of the sulfite, reached a hydrogen concentration ranging from pH 8.6 to 8.8, a degree of alkalinity unfavorable to the development of the Shiga bacillus. By adjustment of the reaction of the medium to pH 7.8–8.0, the inhibiting effect is greatly reduced, and the plates are suitable for the isolation of all the pathogenic members of the typhoid-dysentery class of bacilli.

The agar is prepared in the usual manner, titrated to pH 7.4, flasked in 100 cc. or other suitable amounts, and sterilized.[1] Before the

*Work conducted under a grant from the International Health Board of The Rockefeller Foundation.

[1] This constitutes the stock medium from which all the special media are prepared.

plates are poured the reaction is adjusted to pH 7.8–8.0 by the addition of the requisite amount of N sodium hydroxide solution. The lactose and sulfite-fuchsin solutions are then added under sterile conditions and the plates poured. It was noted that it is sufficient to add 0.5 cc., instead of 1 cc., of the sulfite-fuchsin mixture to 100 cc. of agar, and this amount was found to be preferable in the isolation of dysentery bacilli. The sulfite-fuchsin is prepared by decolorizing 1 cc. of a 10 per cent alcoholic solution of basic fuchsin with 10 cc. of a 10 per cent solution of sodium bisulfite.

Modification of the Brilliant Green Medium.

The brilliant green plate as advocated by Krumwiede, Pratt, and McWilliams,[2] proved to be the most satisfactory of the various inhibitive, selective culture media tested. It lacked, however, the advantage of a sharp differential indicator. A number of indicators were tested and neutral red was found best to answer this purpose. It is not readily reduced by the action of the group of bacteria in question, it does not affect the activity of the dye, and it changes color at the acid zone, beginning at about pH 6.8. The differentiation between fermenting and non-fermenting types is the same as on Endo medium: the *coli* colonies are red or have a reddish center, while the typhoid and paratyphoid colonies are colorless or faintly brownish, owing to the brownish tinge of the plate.

The medium is prepared in the usual manner, care being taken to adjust the reaction to pH 7.0–7.2. This is important, because the reaction of the medium has a marked influence on the activity of the brilliant green, and this hydrogen concentration offers optimum conditions. The lactose, brilliant green, and neutral red solutions are added under sterile conditions just before plating. 0.25 cc. of a 1 per cent water solution of neutral red is added to 100 cc. of agar. The optimum concentration of dye should always be determined with known strains, as directed by Krumwiede. As a rule, 0.3 cc. of a 1: 1,000 solution of the dye added to 100 cc. of agar gives the desired concentration.

[2] Krumwiede, C., Jr., Pratt, J. S., and McWilliams, H. I., *J. Infect. Dis.*, 1916, xviii, 1.

Modification of the Russell Double Sugar Tube Medium.

The Russell double sugar tube,[3] containing 0.1 per cent glucose and 1 per cent lactose with litmus or Andrade indicator, has proved highly serviceable for the rapid differentiation of suspicious colonies fished from Endo or brilliant green plates. The tube enables one to distinguish *coli*, typhoid, and paratyphoid bacilli from one another, but does not differentiate *Bacillus typhosus* from *Bacillus dysenteriæ* or *Bacillus paratyphosus* A from *Bacillus paratyphosus* B. In a previous paper[4] it was reported that such separation is rendered possible by the aid of basic lead acetate. It seemed, therefore, that a combination of the lead acetate with the Russell medium would yield a tube which simultaneously would differentiate all the members of this group. With some slight exceptions this proved to be the case, provided certain precautions, to be mentioned below, are taken in the preparation of the medium.

Preparation of the Medium.

Either meat infusion agar or beef extract agar may be used as a basis for this medium, although sharper reactions are, as a rule, obtained with meat infusion agar. The agar is adjusted to pH 7.4 or neutral to Andrade's indicator, 1 per cent by volume of this indicator is added, and the medium is tubed 5 cc. to a tube and sterilized. The lactose-glucose solution, containing 20 per cent lactose and 2 per cent glucose, is sterilized separately, and 0.25 cc. added in a sterile manner to each tube. The basic lead acetate solution, 0.25 per cent, is also sterilized separately and 1 cc. added to each tube. Both the lactose and the lead acetate solutions may be added before slanting and as soon as the agar is cooled to about 60°C.; otherwise the lead flocculates the peptone.

In order to obtain uniform results, certain details must be taken into consideration. The agar should preferably be sugar-free if meat infusion is used, and as nearly colorless as possible. The browner the medium the poorer are the results, partly because the reaction is

[3] Russell, F. F., *J. Med. Research*, 1911, xxv, 217.

[4] Kligler, I. J., *Am. J. Pub. Health*, 1917, vii, 1042.

masked, and partly because of the breaking down of some of the constituents of the medium itself. Care should be taken to use lactose free from glucose, for the obvious reason that an excess of the hexose vitiates the basic principle of the medium and leads to poorly defined reactions. Still another detail is the character of the slant and the mode of inoculation. The tube[5] should contain enough medium to allow for a butt of at least $\frac{1}{2}$ to $\frac{5}{8}$ inch and a slant of about $1\frac{1}{2}$ inches. The inoculation is made by stabbing the butt near the front and then streaking the slant as the needle is withdrawn from the stab.

If the precautions outlined are followed, it is possible to obtain clear-cut differentiation. *Bacillus coli* reddens the whole tube and breaks up the medium through the gas produced. *Bacillus typhosus* reddens the butt, leaves the slant colorless, and produces browning, particularly near the surface of the stab. The dysentery bacilli redden the butt, like *Bacillus typhosus*, but do not produce browning.

The *paratyphosus* bacilli differ from *Bacillus typhosus* and *Bacillus dysenteriæ* in that they produce gas. Further differentiation between the A and B types is obtained on the basis of the browning. *Bacillus paratyphosus* B and its allied bacilli, *Bacillus enteritidis* and *Bacillus murium*, produce browning, while the A type does not. Occasionally one finds strains of typhoid bacilli which do not cause browning, but they are the exceptions; *Bacillus paratyphosus* B, on the other hand, invariably gives a positive reaction.

CONCLUSION.

The three media as modified—Endo medium, neutral red-brilliant green, and the lead acetate Russell double sugar—have proved in our hands the best combination for the isolation and rapid differentiation of the various organisms belonging to the typhoid-dysentery group of bacilli.

[5] For economy and convenience the Wassermann tube is used instead of the large sized test-tube.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1918,
Vol. xxviii, No. 3, pp. 323–331.]

AN EXPERIMENTAL TEST OF NUZUM'S ANTIPOLIOMYELITIC SERUM.

BY HAROLD L. AMOSS, M.D., AND FREDERICK EBERSON, PH.D.
(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 22 TO 24.

(Received for publication, June 8, 1918.)

In a recent paper[1] we reported a series of experiments with Rosenow's antipoliomyelitic serum, which is prepared in the horse by injections of streptococci derived from cases of poliomyelitis. The present paper deals with a similar test of a serum prepared in the horse by Nuzum[2] also by inoculating streptococci. The two sera—Rosenow's and Nuzum's—while apparently similar, are prepared in somewhat different manner. Thus Nuzum[3] has had the exceptional and on the whole extraordinary experience of cultivating streptococci from the cerebrospinal fluid removed by lumbar puncture from 90 per cent of the cases of epidemic poliomyelitis studied, and he employs these spinal strains in the immunization process. Nuzum's reported results stand alone; no other bacteriologist employing a strict technique has found bacteria either on microscopic examination or in cultures in the cerebrospinal fluid. Perhaps the largest single experience reported is that of Abramson,[4] who published results of the bacteriological study of more than 1,200 fluids, all being negative for bacteria aside from a few instances of obvious contaminations.

However, it is imperative to approach the question of immunological relation of certain streptococci to poliomyelitis without prejudice; and as Rosenow[5] and Nuzum[6] have reported the successful application of their sera, not only in experiments on monkeys, but also in treating human cases of the disease, it is essential that confirmatory and, as far as possible, decisive tests be carried out by others.

We believe that we[1] have performed a decisive test with Rosenow's serum with the result of showing that under the conditions of the experiment it is devoid of protective power when injected intraspinally against experimental infection of monkeys with an active virus of poliomyelitis introduced intravenously. That the conditions of the experiments were not too severe is indicated by the

---

[1] Amoss, H. L., and Eberson, F., *J. Exp. Med.*, 1918, xxvii, 309.
[2] Nuzum, J. W., *J. Am. Med. Assn.*, 1916, lxvii, 1437.
[3] Nuzum, J. W., *J. Am. Med. Assn.*, 1917, lxviii, 24.
[4] Abramson, H. L., *J. Am. Med. Assn.*, 1917, lxviii, 546.
[5] Rosenow, E. C., *J. Am. Med. Assn.*, 1917, lxix, 1074.
[6] Nuzum, J. W., and Willy, R. G., *J. Am. Med. Assn.*, 1917, lxix, 1247.

fact that perfect protection was secured from the serum of monkeys which had recovered from an experimental poliomyelitic infection.

Nuzum[7] has developed a somewhat more elaborate therapeutic technique than Rosenow. Thus he bases his contention that his serum is therapeutically active on three sets of tests: (*a*) the neutralization of the virus *in vitro;* (*b*) the neutralization of the virus *in vivo* by intraspinal injections of the serum; (*c*) the neutralization of the virus *in vivo* by combined intraspinal, intravenous, and intramuscular injections of the serum. Hence, in order to repeat and check Nuzum's experiments, the three modes of administering the serum should be followed.

Through the kindness of Dr. Nuzum we were supplied with 50 cc. of his serum. This proved sufficient for one set of experiments, one monkey being used for each kind of test. The series of tests is therefore a small one; and had the results not been absolutely consistent would have had no great value. But as the results were consistent, and as the control tests with the serum of recovered monkeys were equally definite, no real doubt need be entertained regarding their significance.

EXPERIMENTAL.

The essential condition in dealing experimentally with the virus of poliomyelitis is to possess a specimen of regular and high potency; otherwise an element of irregularity is introduced which involves many separate inoculations. Moreover, the virus should be so active as to produce infection uniformly when small doses of the Berkefeld filtrate are injected intracerebrally. A virus which acts only when relatively large doses of the unfiltered suspension are injected is certain to produce infection irregularly and, in addition, may give confusing results, owing to traumatic and other forms of injury to the brain substance through which paralysis, weakness, and other disturbing effects are produced.

Neutralization in Vitro.

When one minimum lethal dose of virus filtrate is incubated in the presence or absence of complement[8, 9] with 2 cc. of serum from a

[7] Nuzum, J. W., and Willy, R. G., *J. Infect. Dis.*, 1918, xxii, 258.

[8] Levaditi and Landsteiner, *Compt. rend. Soc. biol.* 1910, lxviii, 311. Römer, P. H., and Joseph, K., *Münch. med. Woch.*, 1910, lvii, 568. Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1780. Anderson, J. F., and Frost, W. H., *J. Am. Med. Assn.*, 1911, lvi, 663.

[9] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1918, xxviii, 11.

monkey which has had experimental poliomyelitis and recovered, the virus is rendered non-infective for a monkey on intracerebral inoculation. Normal serum[9] does not possess this power.

Three experiments were carried out in precisely the same manner, comparing Nuzum and Willy's serum with normal horse serum and immune monkey serum. To 2 cc. of the serum to be tested there was added 0.2 cc. of a Berkefeld filtrate of a 5 per cent suspension of glycerolated active poliomyelitic monkey cord. The mixture was incubated for 2 hours at 37° C. and placed at 4°C. for 16 hours, and then injected intracerebrally into a normal monkey.

Monkey A (*Macacus rhesus*), *Control.*—Apr. 12, 1918. Injected intracerebrally the incubated mixture resulting from the addition of 0.2 cc. of virus filtrate to 2 cc. of normal horse serum. Apr. 16. Excited and slow. Apr. 17, a.m., prostrate, moribund; p.m., died.

Autopsy showed characteristic changes of poliomyelitis in the brain and cord.

Monkey B (*Macacus rhesus*).—Apr. 12, 1918. Injected intracerebrally the incubated mixture resulting from the addition of 0.2 cc. of virus filtrate to 2 cc. of Nuzum and Willy's serum. Apr. 19, a.m., marked ataxia, both arms paralyzed (Fig. 1); p.m., prostrate, etherized.

Microscopic examination of the nervous tissues showed typical diffuse and extensive poliomyelitic lesions in medulla and spinal cord; marked perivascular infiltration (Fig. 2), necrosis, neurophagocytosis of the ganglion cells, and focal hemorrhages in the anterior horn of the cord.

Monkey C (*Macacus rhesus*), *Immune Control.*—Apr. 12, 1918. Injected intracerebrally the incubated mixture resulting from the addition of 0.2 cc. of virus filtrate to 2 cc. of serum drawn from a monkey which had been paralyzed from experimental poliomyelitis and recovered. The monkey remained well.

The results show that Nuzum and Willy's serum, under the conditions of the experiment, possesses no more neutralizing power for poliomyelitic virus *in vitro* than does normal horse serum.

Neutralization in Vivo.

Nuzum and Willy's serum was subjected in the following experiments to the same test applied to Rosenow's antipoliomyelitic serum. This consists in the injection of a large dose of the virus intravenously into a monkey, and the repeated intraspinal injection of the serum to

be tested. Flexner and Amoss[10, 11] have shown that under these conditions a serum possessing no neutralizing properties sets up inflammatory changes, thus opening the way for the virus to pass from the blood stream into close relation with the meninges and cause infection. Immune serum, on the other hand, while causing the same inflammatory changes, neutralizes the virus as it passes through and prevents infection.

Monkey D (*Macacus rhesus*), *Control.*—Apr. 9, 1918, 5 p.m. Injected intraspinally 2 cc. of normal human serum. Apr. 10, 11 a.m. Injected intravenously 50 cc. of a centrifuged 5 per cent suspension of fresh poliomyelitic cord in isotonic salt solution. 11.10 a.m. Injected intraspinally 2 cc. of normal human serum. Apr. 11, 12 m. Intraspinal injection of 2 cc. of pooled serum from monkeys recovered from experimental poliomyelitis. The dose of serum was repeated on the 2 following days. The monkey remained well.

Monkey E (*Macacus rhesus*).—Apr. 9, 1918, 4.30 p.m. Injected intraspinally 2 cc. of Nuzum and Willy's antipoliomyelitic serum. Apr. 10, 11.15 a.m. Injected intravenously 50 cc. of a centrifuged 5 per cent suspension of fresh poliomyelitic cord in isotonic salt solution. 11.25 a.m. Injected intraspinally 2 cc. of Nuzum and Willy's serum. The intraspinal injection of 2 cc. of the serum was repeated daily on the 3 succeeding days. Apr. 16. Slow; excitable. Apr. 17. The same. Apr. 18. Slow; tires easily. Apr. 19. Prostrate (Fig. 3). 3.10 p. m. Died.

Autopsy and microscopic examination of the tissues showed lesions of poliomyelitis, chiefly in the medulla and cervical enlargement of the cord. They consisted of the usual perivascular infiltrations, which were of moderate severity, and of focal infiltrations of cells with polymorphous and fragmented nuclei. The focal infiltrations were sometimes near and sometimes away from blood vessels and were frequently in association with hemorrhages of considerable size. Moreover, nerve cell degeneration and neurophagocytosis had been progressing actively in the medulla (Fig. 4) and in the cervical portion of the cord. These lesions indicate a severe poliomyelitis. The number of focal hemorrhages (Fig. 4) and their size are unusual in experimental poliomyelitis, although they are commonly found in human poliomyelitic nervous tissues.

The experiments show that while the immune monkey serum completely neutralizes the virus as it passes through into the meninges and thus prevents infection, the serum of Nuzum and Willy possesses no such power, but acts in the same manner as normal horse serum.

[10] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.
[11] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 525.

Curative Experiments.

Nuzum and Willy make the claim that their serum possesses curative properties against the virus in experimental poliomyelitis in monkeys. In order to test this property an experiment was made comparable with their recorded test with Monkey 25 of their series. Their dose of virus given intracerebrally was 1 cc. of a 5 per cent suspension of poliomyelitic cord. In the experiment about to be recorded only 0.5 cc. of a Berkefeld filtrate of a 5 per cent suspension of poliomyelitic monkey cord was used. Thus the test in our hands was apparently less severe; however, the result is decisive.

Monkey F (*Macacus rhesus*).—Apr. 11, 1918, 2.55 p.m. Injected intracerebrally under ether anesthesia 0.5 cc. of a Berkefeld filtrate of a 5 per cent suspension of glycerolated poliomyelitic monkey cord. 3 p.m. Injected intravenously 15 cc. and intraspinally 2.5 cc. of Nuzum and Willy's serum. Apr. 13. Injected intramuscularly, left gluteal region, 10 cc. of the serum. Apr. 14. Injected 10 cc. of the serum intramuscularly. Apr. 16. Ataxic; arms and legs weak; coarse tremor present. Apr. 17, 8 a.m. Found dead.

Autopsy and microscopic examination of the brain and cord showed typical diffuse lesions of poliomyelitis in the spinal cord and medulla. (Fig. 5.) There were extensive necrosis of the gray matter in the cord and marked typical perivascular infiltration in the intervertebral ganglia, with hyaline degeneration and neurophagocytosis of the ganglion cells. The medulla showed neurophagocytosis of nerve cells, focal infiltration of cells, of which many were polymorphonuclear, due probably to the injection of horse serum.

DISCUSSION.

The significance of the experiments is self-evident. Thus, while the serum of the recovered monkeys completely neutralizes the virus of poliomyelitis *in vitro* or as it passes from the blood into the meninges after an aseptic inflammation has been induced in the latter, the serum prepared in the horse by Nuzum and Willy by means of injections of streptococci exhibited no neutralizing power whatever as far as could be detected. Indeed, the indications are that the latter serum acts in the manner of normal horse serum and as such rather promotes than prevents experimental poliomyelitic infection.

This, the main question raised by the experiments, having been answered, several subsidiary questions may now be considered. The

first one of the latter relates to the character of the experimental evidence on which Rosenow[12] and Nuzum[6] base their claim that the antistreptococcic serum is neutralizing for the virus of poliomyelitis. It is significant that Rosenow[12] uses the expression "appears to have developed neutralizing, protective and curative power against the virus of poliomyelitis." The tests in monkeys can be made so definite that there need be no room left for doubt as to what the results imply. There are few pathogenic organisms which may be made to yield laboratory results as clear-cut as the virus of poliomyelitis.

The basis of Nuzum's conclusion is also not definite. He claims for his serum not merely neutralizing, protective power, but curative properties and the ability to immunize monkeys passively against subsequent intracerebral inoculation of active virus suspension. Altogether the number of criteria which enter into his calculations makes it difficult to arrive at a decision. The source of this uncertainty may reside in the quality of the virus which Nuzum employed. As he injected 1 cc. of an unfiltered emulsion and even then failed in some instances to secure infection, the potency must have been low. As we have already pointed out, a virus with low virulence gives such irregular results as to make accurate deductions difficult or impossible to draw. In our opinion, therefore, Nuzum's experimental evidence is inconclusive.

Both Rosenow[5, 13] and Nuzum[6] have treated human cases of poliomyelitis with their antistreptococcic sera. The mode of application differs. Thus Rosenow injects the serum intravenously, and Nuzum uses the combined intraspinal and intravenous injection and sometimes intramuscular injections. The question as to the value of the evidence which is provided by the treatment of human cases of poliomyelitis in the preparalytic stages, so called, in which, apparently, the therapeutic results are supposed to be striking, cannot now be answered. Our knowledge of the evolution of the preparalytic cases is still in its infancy. The wide employment of lumbar puncture, in the last 2 years, for diagnostic purposes, is giving us for the first time data on which eventual conclusions may come to be based. It would

[12] Rosenow, E. C., *J. Am. Med. Assn.*, 1917, lxix, 261, 1074.
[13] Rosenow, E. C., *J. Infect. Dis.*, 1918, xxii, 379.

be premature to undertake to interpret those data now. On the other hand, little evidence has been adduced that the antistreptococcic serum injections have affected the mortality in the frankly paralytic cases, halted the advancing paralysis, or brought about a more rapid and complete retrocession of the existing paralyses. At the best, therefore, the case for the serum, as far as human therapy goes, should be considered unproved.

There is, however, another subsidiary aspect of the subject. Streptococcus infection as a concomitant or terminal or agonal infection in epidemic poliomyelitis would now seem not to be uncommon. It is not yet clear just when the invasion of streptococci into the nervous system takes place. The fact that the cerebrospinal fluid, withdrawn under sterile conditions, practically never contains the streptococci, would seem to indicate that the antemortem invasion is small or agonal. The point is very difficult to decide in human cases. On the other hand, it is one that should be easily decided experimentally. This Smillie[14] has attempted to do. Streptococci may be cultivated from the brain[15] and cord and other viscera[14] of monkeys which have succumbed to experimental poliomyelitis. When the spinal cord and medulla carrying streptococci are placed in 50 per cent sterile glycerol, the streptococci survive for weeks. On reinoculating this material intracerebrally into monkeys, the streptococci, if still alive, either merely survive or multiply somewhat alongside the poliomyelitic virus. Hence they can be recovered from the inoculated brain tissue. But if inoculation is made with a filtered virus free of streptococci, then, as Smillie has found, the presence or absence of those organisms is determined in great part by the stage of the disease and the state of the animal at the time the postmortem examination is performed and cultures are taken. An animal etherized when paralyzed and still strong tends not to show the presence of streptococci, while a moribund or already dead animal often does show their presence. In

[14] Smillie, W. G., *J. Exp. Med.*, 1918, xxvii, 319.

[15] Mathers, G., *J. Am. Med. Assn.*, 1916, lxvii, 1019; *J. Infect. Dis.*, 1917, xx, 113. Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.* 1916, lxvii, 1202; *Science*, 1916, xliv, 614; *J. Am. Med. Assn.*, 1917, lxviii, 280. Nuzum, J. W., and Herzog, M., *J. Am. Med. Assn.*, 1916, lxvii, 1205. Nuzum J. W., *ibid.*, 1916, lxvii, 1437; 1917, lxviii, 24.

other words, Smillie's view,which has still to be confirmed, would place the streptococcal invasion in poliomyelitis among the agonal and not the concomitant infections.

It is on the basis of a possible concomitant streptococcus infection of real importance for the termination of the disease that the employment of an antistreptococcic serum in poliomyelitis may still be urged, once it is proved that it has no specific action on the virus of poliomyelitis. Even for this purpose we should need far more knowledge than we now possess to justify its employment.

The value, in general, of antistreptococcic sera in combating streptococcus infections is unproved. Streptococci have not been cultivated from cases of poliomyelitis in man during life. Many, chiefly negative, results have been obtained with cultures from the cerebrospinal fluid; and it is in our opinion desirable to make many cultures from the blood before resorting to the intravenous injection of antistreptococcic serum on a large scale on the assumption of a streptococcus infection playing an essential part in the pathology of poliomyelitis.

CONCLUSIONS.

The antistreptococcic serum of Nuzum and Willy has failed to show in the monkey neutralizing or therapeutic power when applied by their methods against small doses of the virus of poliomyelitis. Under the same conditions the serum of monkeys recovered from experimental poliomyelitis proved neutralizing and protective.

The experimental and other evidence adduced by those who regard the streptococcus as playing an essential part in the pathology of epidemic poliomyelitis and the antistreptococcic sera as exhibiting therapeutic properties for man and monkeys is regarded as imperfect and inconclusive.

EXPLANATION OF PLATES.

PLATE 22.

FIG. 1. Monkey B. 7 days after the intracerebral injection of the incubated mixture of 0.2 cc. of virus filtrate and 2 cc. of Nuzum and Willy's serum.

FIG. 2. Monkey B. Cervical enlargement of the cord showing perivascular infiltration. × 165.

PLATE 23.

FIG. 3. Monkey E. 9 days after the intravenous injection of virus. This monkey was treated with five intraspinal injections of 2 cc. each of Nuzum and Willy's serum.

FIG. 4. Monkey E. Medulla, showing perivascular infiltration, hemorrhage, and neurophagocytosis. × 192.

PLATE 24.

FIG. 5. Monkey F. Medulla, showing congestion, necrosis of gray matter, cell degeneration, and neurophagocytosis. × 240.

FIG. 1.

FIG. 2.

(Amoss and Eberson: Nuzum's antipoliomyelitic serum.)

FIG. 3.

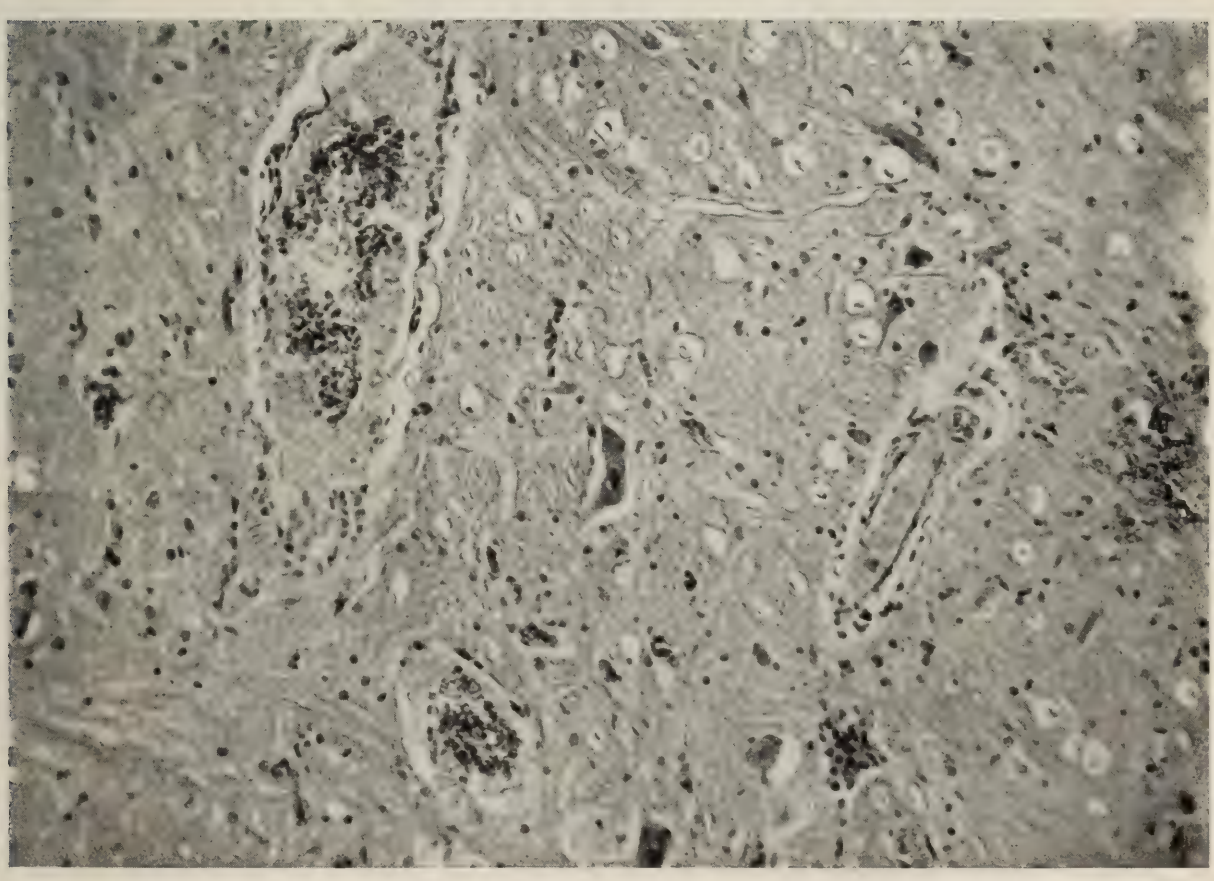


FIG. 4.

(Amoss and Eberson: Nuzum's antipoliomyelitic serum.)

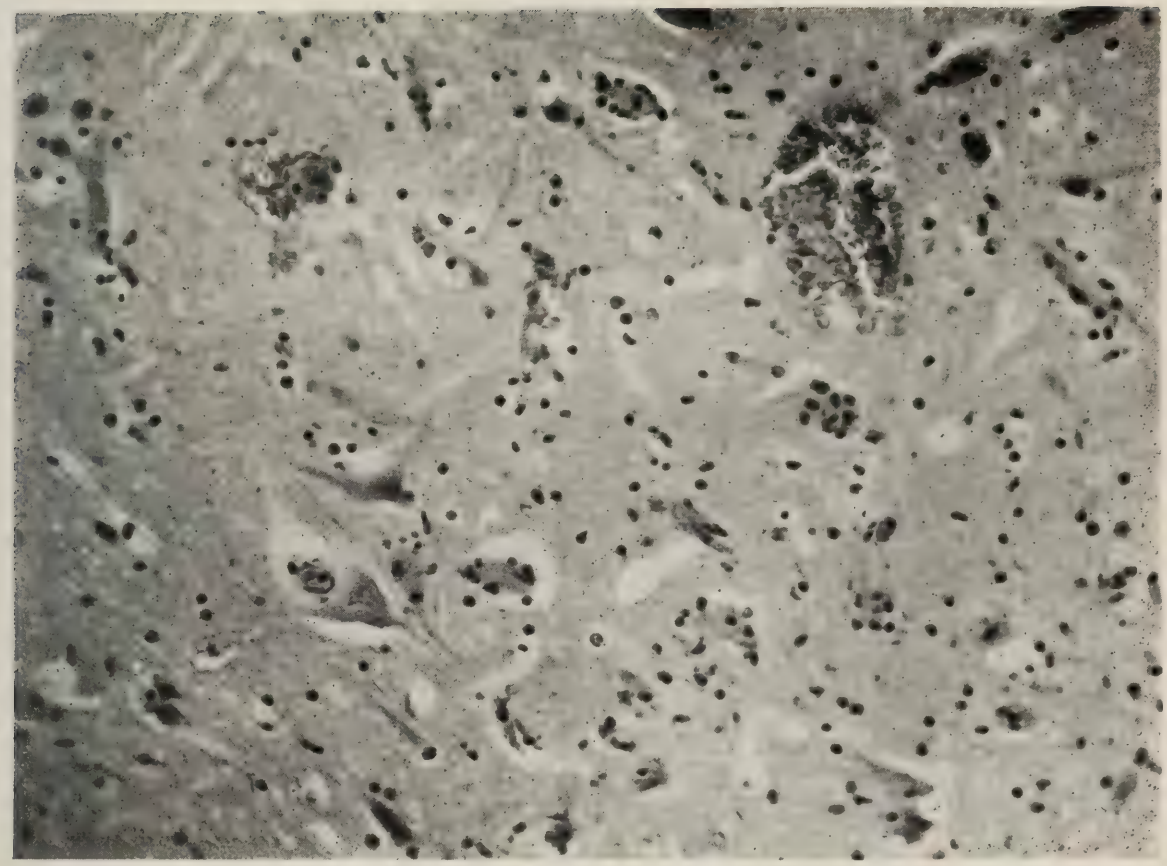


FIG. 5.

(Amoss and Eberson: Nuzum's antipoliomyelitic serum.)

[Reprinted from The American Journal of Public Health, August, 1918, Vol. viii, No. 8, pp. 590–593.]

SURVIVAL OF TYPHOID BACILLI IN SOUR MILK.

By PENELOPE MARSH.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 10, 1918.)

Certain communities in our population make a practice of drinking milk permitted to undergo souring spontaneously. The question arose whether it is necessary to subject the raw milk used for souring purposes to preliminary pasteurization in order to insure the destruction of the typhoid-paratyphoid group of bacilli, or whether any of those bacteria which might be present would not be destroyed by the acids developed in the souring process. In order to answer this question a series of tests was arranged, the results of which are given in the tabulations of this paper.

The subject of investigation is not wholly a new one, although the practical point of departure of this study may be peculiar. A brief survey of the earlier literature on the subject includes the following:

Cautley (1) found that typhoid bacilli which had been added in large amount to milk could be demonstrated in the living state for several days, and that the viability of these organisms depended on the number and character of the contaminating bacteria. In sterilized milk the typhoid bacilli were found to survive as long as four months, according to Bolley and Field (2).

Fraenkel and Kister (3) added an enormous dose (2, $\frac{1}{2}$, and $\frac{1}{3}$ loops) to 10 cc. of buttermilk. At room temperature the typhoid bacilli remained alive for three days, while at incubator temperature they died out after twenty-four hours.

Bruck (4) endeavored to approximate natural conditions and recovered the bacilli from sour cream for ten days after its separation from artificially infected milk. He also isolated them from buttermilk for the same period of time and from butter, prepared from infected cream, for twenty-seven days.

Bassenge (5) states that the typhoid bacilli are killed when the acid production reaches 0.3 per cent to 0.4 per cent and is maintained there for twenty-four hours. This opinion is not shared, however, by many other workers.

Krumwiede and Noble (6) say that "the typhoid bacillus is gradually killed in sour cream by the acids produced, the rate of destruction being proportional to the degree of acidity and the number of typhoid bacilli present." They find that typhoid bacilli are killed in about four days in a moderately contaminated cream, but that they will live longer if initial multiplication has occurred.

Lactic acid in varying amounts was added to milk containing typhoid bacilli by Trillat and Fouassier (7), and its inhibitive effect demonstrated. They also made a comparative study of the rate of multiplication of typhoid bacilli in broth and milk. In milk multiplication began at once, but in broth no development was noticed for twenty-four hours.

EXPERIMENTAL.

In order to determine the viability of the typhoid and certain related bacilli in milk undergoing natural acidification, the following tests were made: The method was to inoculate the milk samples and then to determine viability by culture and acidity by titration at specified intervals afterwards, the samples being kept at room temperature, which on the average was 15°C. (60° F.).

The milk was that purchasable in New York City and had, therefore, in the case of full milk, been pasteurized. The samples containing typhoid and paratyphoid bacilli were plated on brilliant green agar (containing 0.3 cc. of 1:1000 solution of the dye per 100 cc. nutrient agar) while the samples containing the dysentery bacillus were plated on Endo medium. The platings were approximately daily, as was the titration of acidity. The latter was performed as follows: 5 cc. of the milk were added to 45 cc. distilled water and heated to the boiling point, and 1 cc. 0.5 per cent phenolphthalein added as indicator. The N/10 NaOH was allowed to run into the fluid from a burette until it turned a delicate pink which did not disappear on heating. The acidity was measured in amounts of NaOH. At the outset of the experiment the milk samples to which

the *B. typhosus* and *paratyphosus* were added was 0.7, and the sample to which *B. dysenteriæ* (Flexner) was added was 0.8.

To each of three-quart bottles of "B" grade milk was added one platinum loop of a 24-hour agar culture of *B. typhosus*, *paratyphosus*, or *dysenteriæ* (Flexner). The daily routine of culturing was first to stir each bottle with a sterile glass rod and then to plate one platinum loop of the sample. After incubating the plate at 37°C. over night, the approximate number of colonies was recorded, and an isolated colony was stained by Gram, planted on Russell's double sugar agar slant, and agglutinated with a specific serum.

Before the inoculation with the specific bacilli a loopful of each sample of milk was plated. The two bottles subsequently inoculated with *B. typhosus* and *paratyphosus* yielded in the brilliant green plates 15 to 20 colonies of large Gram positive and Gram negative bacilli and small Gram negative bacilli. The Endo plates yielded half a dozen staphylococcus colonies.

On the other hand, the plates made immediately after the inoculation gave heavy growths in pure culture of *B. typhosus* and *paratyphosus* and a heavy growth of *B. dysenteriæ* in which two colonies of staphylococci were detected. Table I summarizes the result of this test. The + + indicate abundant growth, + moderate growth, ± a few colonies.

The next experiment was made with the typhoid bacilli in Grade "B" pasteurized milk and in buttermilk. The preliminary titrations of four bottles of "B" milk were 1.0, 1.1, 1.1, and 1.2, and of the buttermilk 3.5, 4.2, 4.2, and 4.4.

The inoculations were performed as indicated. Tables II and III show briefly the result.

Finally, the test with buttermilk was repeated and the results of the last test, using four separates bottles, are shown in Table IV.

TABLE I.

Viability of B. typhosus, B. paratyphosus, and B. dysenteriæ in Milk.

	Nov. 6	Nov. 7	Nov. 8	Nov. 9	Nov. 10	Nov. 12	Nov. 13	Remarks.
B. typhosus								
acidity.....	.7	.7	1.4	2.6	2.6	2.8	4.9	Nov. 13, plate showed 2 extraneous colonies and no *B. typhosus*.
culture.....	0	++	++	++	+	±	0	
B. paratyphosus								
acidity.....	.7	.7	1.0	3.1	4.0	4.3	4.8	Nov. 10, contaminating colonies. Nov. 12, no *B. paratyphosus*.
culture.....	0	++	++	++	+	0	0	
B. dysenteriæ								
acidity.....	.8	.8	1.4	2.9	4.2	4.2	3.6	Nov. 7, two staphylococcus colonies. After Nov. 10 no *B. dysenteriæ*.
culture.....	0	++	++	++	0	0	0	

TABLE II.

Viability of B. typhosus in Fresh Milk.

	Nov. 26	Nov. 27	Nov. 28	Nov. 30	Dec. 1	Dec. 3	Remarks.
Bottle 1							
acidity.....	1.1	1.4	4.6	5.2	6.1		Nov. 30, heavy contamination. No B. *typhosus* present.
culture.....	0	+	++	0	0	0	
Bottle 2							
acidity.....	1.4	1.7	3.0	5.6	6.6		Heavy contamination, Nov. 30. No *B. typhosus* present.
culture.....	0	0	+	0	0	0	
Bottle 3							
acidity.....	.9	1.1	4.4	6.0	6.3		Heavy contamination, Nov. 30. No *B. typhosus* present after Nov. 27.
culture.....	0	+	0	0	0	0	
Bottle 4							
acidity.....	1.0	1.1	4.0	5.1	5.3		Heavy contamination, Nov. 30. No. *B typhosus* present.
culture.....	0	+	++	0	0	0	

TABLE III.

Viability of B. typhosus in Buttermilk.

	Nov. 14	Nov. 15	Nov. 16	Nov. 17	Nov. 18
Bottle 1					
acidity	3.5	4.3	5.0	4.8	6.6
culture	0	+	+	0	0
Bottle 2					
acidity	4.2	4.5	6.4	5.5	6.9
culture	0	+	+	+	0
Bottle 3					
acidity	4.2	4.3	5.5	5.8	6.3
culture	0	+	+	+	0
Bottle 4					
acidity	4.4	4.7	5.0	5.2	5.5
culture	0	+	+	+	0

TABLE IV.

Viability of B. typhosus in Buttermilk.

	Nov. 26	Nov. 27	Nov. 28	Nov. 30	Dec. 1	Remarks.
Bottle 1						
acidity	4.5	4.7	4.8	4.5	4.4	
culture	0	+	0	0	0	
Bottle 2						
acidity	4.2	4.2	4.2	4.2	4.2	
culture	0	0	0	0	0	
Bottle 3						
acidity	3.4	3.8	3.8	4.2	5.0	Slight contamination, Nov. 30
culture	0	+	0	0	0	
Bottle 4						
acidity	3.1	3.7	4.3	4.6	4.6	Slight contamination, Nov. 30
culture	0	+	0	0	0	

DISCUSSION.

Table I shows the effects of acidity as such on the multiplication and survival of *B. typhosus*, *paratyphosus*, and *dysenteriæ* (Flexner) in milk. Few contaminating organisms which grew on aerobic plates of brilliant green agar or Endo appeared to complicate the result. With an acidity represented by about 4.0 cc. of N/10 NaOH, the typhoid and paratyphoid bacilli disappear. The dysentery bacilli are more sensitive, disappearing one day earlier than the others. Table II is somewhat more complicated because it introduces the factor of contaminating organisms which grow on the special media. And yet it is only after an acidity of 4.0 is reached that the latter appear as coincidently *B. typhosus* disappears. It appears, therefore, that the latter were not so much overgrown as destroyed by the degree of acidity. The two tests with buttermilk (Tables III and IV) indicate essentially the same thing. No multiplication, but merely speedy destruction, occurred in the samples of Table IV at the original acidity of 3.8 to 4.7; while in three of the four samples of Table II survival of the bacilli (without multiplication) persisted for two days, during which the acidity changed from the initial 4.3–4.7 to 5.0–6.4.

The tests indicate that *B. typhosus* is sensitive to acidity and is usually destroyed in milk in a short time at about the degree of acidity occurring in fresh buttermilk as obtained in the New York market. Buttermilk would seem to be free of danger as a means of distributing *B. typhosus* or *paratyphosus*, or *B. dysenteriæ* in the ordinary course of events.

REFERENCES.

1. Cautley, Edmund, Rept. Med. Off., Local Govt. Bd., London, 1896–7, 243.
2. Bolley, H. I., and Field, W., Centralbl. f. Bakt., Abt. II, 1898, iv, 881.
3. Fraenkel, E., and Kister, J., München. med. Wchnschr., 1898, xlv, 197.
4. Bruck, Carl, Deutsch. med. Wchnschr., 1903, xxix, 460.
5. Bassenge, R., Deutsch. med. Wchnschr., 1903, xxix, 675.
6. Krumwiede, C., Jr., and Noble, W. C., Am. Jour. Pub. Health, 1914, iv, 1006.
7. Trillat, A., and Fouassier, W., Compt. rend. de l'acad. d. Sc., 1916, clxii, 849.

[Reprinted from The Journal of General Physiology, September 20, 1918, Vol. i, No. 1, pp. 23–32.]

THE ANTAGONISM BETWEEN THYMUS AND PARATHYROID GLANDS.

By EDUARD UHLENHUTH.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 16, 1918.)

INTRODUCTION.

It is well known that the extirpation of the parathyroids in mammals is followed by tetanic convulsions. This led to the conclusion that certain tetany-producing toxins are formed in the body, which in normal animals must be either removed or turned into non-toxic substances by the parathyroid glands. The origin of these toxic substances, however, is unknown. Though tetanic convulsions and other symptoms of tetany have been reported repeatedly to occur after injection of thymus extracts, in the frog as well as in mammals,[1] no attention has been paid to this fact as possibly containing the solution of the problem in which organ the tetany-producing substances might be manufactured. The experiments presented in this article seem to indicate that they are contained in and produced by the thymus gland. This harmonizes with the well known fact that tetany is a disease of infants.

In the spring of 1916, about thirty salamander larvæ of the species *Ambystoma maculatum*[2] were fed exclusively on calf's thymus. Each single larva after some time began to suffer from severe tetanic attacks. Since the larvæ of salamanders do not possess parathyroids this observation seemed to be of considerable interest, and, in the fall of 1916, calf's thymus was fed to larvæ of another species of sala-

[1] See Biedl, i, 279, 301 ff.

[2] In the terminology to be employed in the classification of amphibians the nomenclature as worked out by Stejneger and Barbour in their new check list was applied here. According to this the old species *Amblystoma punctatum* corresponds to the new species *Ambystoma maculatum*.

manders, *Ambystoma opacum;* again each single individual after a certain time came down with tetanic attacks. In 1917, the experiments were repeated with the same result on both species. It is, therefore, evident that the thymus gland contains a substance which produces tetanic convulsions in the larvæ of *Ambystoma maculatum* and *opacum.*

Tetanic Symptoms.

The tetanic symptoms exhibited by salamander larvæ fed with thymus closely resemble the symptoms produced by parathyroidectomy in mammals.[3] The first attacks in the larvæ are confined to the hind portions of the body. In general, this part of the body suffers more than any other during the entire tetanic period, and when the acute attacks become less and less frequent and severe it is again the hind portions of the body which alone are attacked. Within several days after the onset of acute tetany the entire muscular system exhibits severe clonic convulsions. Each single attack begins at the tail and spreads toward the head; the tremors are severest in the legs and in the muscles of the lower jaw. During each attack the mouth is thrown widely open. When the attack begins the animals are thrown on one side. Besides the clonic convulsions a tonicspasm of the entire body is observed; the legs are stretched out and the body is bent with its concavity towards the back. During the attack the animals discharge much air and the vessels of the skin become very red.

In the early stages of the disease the convulsions are induced only upon stimulation which is best effected by removing the larvæ from the water, placing them on filter paper, and pinching the legs or the tail slightly. Each attack lasts only a short time, the larvæ recovering after from 2 to 3 minutes. Later any attempt of the larvæ to swim or to snap at a piece of food suffices to induce an attack and some individuals may float for several days on one side, being rigid from tonic spasm.

8 to 14 days after the first attack the hind legs begin to show signs of a permanent tonic spasm; the legs are stretched backward and become twisted around their longitudinal axis with the inner surface pointing upward; the feet follow this movement and finally the fore legs undergo a similar change.

[3] See Biedl, i, 79 ff.

Relation between Tetany and Development.

Tetany cannot be produced by thymus feeding before a certain developmental stage is reached by the larva; namely, that at which probably the secretive power of the animal's own thymus glands is established. This stage of development corresponds to that of the full development of the toes of the larvæ.

Six larvæ of *Ambystoma opacum* (*opacum*, 1917, B), from North Carolina, hatched in the laboratory from eggs of the same female, were kept at approximately 25°C. and fed on calf's thymus from the 19th day after hatching. The animals were tested for tetany regularly on a definite day every week, for the purpose of recording the percentage of animals suffering from tetany; clonic convulsions of the entire body as well as of the hind portion only were considered as indication of tetany. No animal suffered from tetany when examined the first time after the commencement of the thymus feeding. With the progress of development (as indicated by the development of the toes) the percentage of animals suffering from tetanic convulsions increased and reached a first maximum at the time when all toes were developed. No animal had convulsions until all the toes of the fore legs and at least the third toe of the hind limbs were developed.

In a second series of experiments, with eight *Ambystoma opacum* larvæ (*opacum*, 1916, B) hatched from eggs of the same female and kept at approximately 25°C., thymus feeding was started on the 14th day after hatching. None of the larvæ of this series showed any signs of tetany before the full number of toes had developed. In a third series (*Ambystoma opacum*, 1916, D) consisting of eight larvæ from eggs of the same female and of the same age as the larvæ of the previous series, this becomes still more evident. Though the larvæ of Series D. 1916, were fed on thymus simultaneously with Series B, 1916, convulsions developed 4 weeks later than in Series B, 1916. Development was retarded by keeping the larvæ in low temperature (15°C, approximately).

In *Ambystoma maculatum* similar results were obtained. In one instance (*Ambystoma punctatum*, Series K, 1917) six larvæ, all hatched from eggs of the same female and kept at approximately 25°C., were fed on thymus exclusively from the 13th day after hatching. The first acute attacks were observed 5 weeks after hatching.

Similarly it was found in *Ambystoma opacum* as well as in *Ambystoma maculatum* that the acute tetanic attacks disappear entirely after a certain stage is reached, though the animals continued to be fed exclusively on thymus gland. When the larvæ reach a stage in which they resemble normal larvæ ready for metamorphosis, it becomes more and more difficult to induce an acute tetanic attack and no animal ever showed such attacks after it had metamorphosed, in spite of thymus feeding.

Thymus Gland Contains a Tetany-Producing Substance.

The question arises: Why does the thymus gland when fed to salamander larvæ cause tetany only during a certain developmental period, and why is it ineffective before and after this period? In order to give an answer to this question it was necessary to make a histological examination of the larvæ.

As regards the beginning of tetany several larvæ which had been fed thymus were examined histologically just before and after the beginning of tetany. In a number of *Ambystoma opacum* larvæ of the same age and hatched from eggs of the same female (*Ambystoma opacum*, 1917) tetany was observed in a few individuals as early as 4 weeks after hatching; in these few individuals, however, it did not reappear until 6 to 7 weeks after hatching, and at this time the majority of the larvæ developed tetany. At this time larvæ of this set with and without tetany were killed and preserved for examination to find in which point they differed. A preliminary microscopical study of the anatomy and histology of one tetanic and two pretetanic larvæ demonstrated a noteworthy difference in regard to the developmental state of the thymus glands, but in no other respect.

It was found that the larvæ which had not yet developed tetany had smaller thymus glands than the larva which had already fallen a victim to tetany. The thymus glands of the latter larva are more than twice as large as those of the pretetanic larvæ, though the difference in length of the entire animals is only 33 per cent, the tetanic specimens being the larger ones. This is shown in Fig. 1 in which the three left thymus glands of a pretetanic larva (left vertical row)

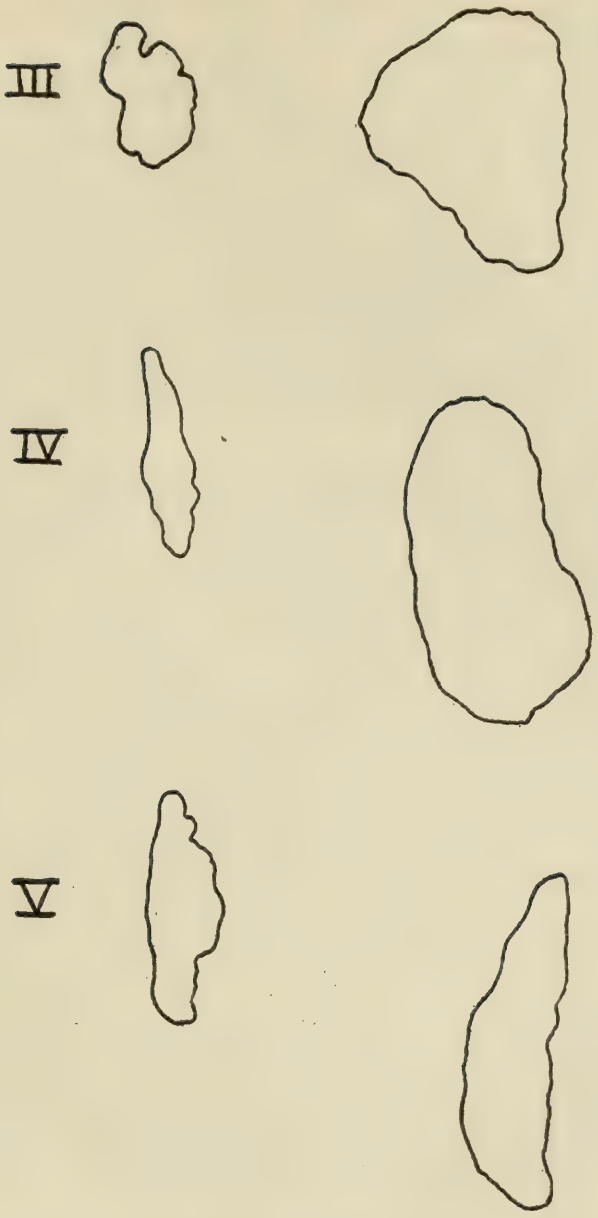


FIG. 1. Comparison between size of the primordial thymus of a pretetanic larva of *Ambystoma opacum* (left vertical row) and a larva of the same species shortly after the beginning of acute tetanic convulsions (right vertical row). It will be seen at a glance that the thymus glands of the latter animal are much larger than those of the non-tetanic larva. The drawings were made with a camera lucida at 490 linear magnification from the largest cross-section through each of the glands.

and of the tetanic larva (right vertical row) are drawn at the same magnification.[4]

Still more significant are the differences in structure. In the pretetanic larvæ the thymus glands represent small accumulations of purely epithelial cells. They do not show any kind of differentiation. Their epithelial character is indicated also by the presence of considerable quantities of pigment granules such as are found in the epithelium of the pharyngeal pouches from which the glands have separated. In most of the glands there are no mitoses to be found at all, while in some there are one to two mitoses present. No membrana propria is formed.

Compared with the thymus of the pretetanic larvæ, in which none of the characters of a true thymus has developed, five of the glands of the tetanic larva show some of the aspects of a real thymus in as far as they contain elements which are characteristic of that organ. Again no membrana propria is formed, but the glands consist of a variety of cells. Leaving aside the question as to whether or not the newly developed cell elements are of mesenchymal origin,[5] it is sufficient to notice that several kinds of cells now constitute these glands; two of them are recognized clearly as the main elements making up the fully developed thymus of an *Ambystoma opacum* larva.

[4] The first and second pairs of the thymus glands have disappeared already in this stage from both the pretetanic and tetanic larvæ, and only the third, fourth, and fifth pairs are present. According to Maximow and to Baldwin the first and second pairs are found absent already in larvæ of from 11.5 to 13.0 mm. in length.

[5] Concerning the development of the amphibian thymus glands and the origin of their cell elements see the papers by Maximow, by Dustin, and by Baldwin. It should be mentioned that the development of the thymus glands in *Ambystoma opacum* was found by the writer to correspond in the main with the description as given by Maximow and Baldwin for *Ambystoma tigrinum* and *Ambystoma maculatum* respectively. The size of the larvæ, however, corresponding to the various developmental stages of the glands was found to be much more variable and in general larger. In a normal stock animal of *Ambystoma opacum*, for instance, the right gland of the third pair was found to be still connected by an epithelial stalk with the brancho-pharyngeal epithelium and of purely epithelial character, though the larva has a length of over 37 mm., while Maximow and Baldwin found the glands fully separated in larvæ of from 10 to 12 mm.; Maximow saw the commencement of mesenchymal immigration in larvæ of about 10 mm. in length, and Baldwin in larvæ of 19 to 20 mm. in length.

Correspondingly the epithelial character of the glands is lost; this finds its expression also in the fact that pigment granules have disappeared altogether. In addition a considerable number of mitoses may be found, which mostly seem to be cell divisions of the newly arrived elements. Of particular importance is the fact that in one of the glands of the fifth pair, which I found to be frequently ahead of the third and fourth pairs in development, the cell plasma of a number of cells stains pink, and here one nucleus which also is stained pinkish exhibits a swollen appearance, characters which are ascribed by Maximow to the formation of the medulla of the thymus.

The thymus glands of the pretetanic larvæ are therefore mere accumulations of epithelial cells and do not possess any structures to indicate that they would be capable of exerting any of the functions of a thymus, while in the tetanic larva at least some of the glands resemble a true thymus and probably have commenced to behave as such with regard to function. From these observations we may conclude that the thymus feeding does not begin to call forth tetany in salamander larvæ until the thymus glands of the larvæ themselves are able to secrete. This suggests that tetany is the effect of the combined action of the secretion of certain substances by the thymus glands of the larvæ and of substances introduced into the larva by feeding it with the thymus gland from other animals.

Prevention of Tetany by the Parathyroids.

It is well known that salamander larvæ do not possess parathyroids; these glands develop during metamorphosis. Though the end of the tetanic period coincides approximately with the development of the parathyroid glands, yet larvæ which had ceased to show convulsions for some time apparently possessed no parathyroids when examined histologically. It is, however, possible that in spite of this in such larvæ certain processes had commenced which would soon have led to the formation of these glands and that these processes were already able to check tetany.

A connection between the development of the parathyroids and the cessation of tetany seems probable on account of the fact that tetany

was never observed after metamorphosis and after the development of the parathyroids. This suggestion finds support in the fact that in the larvæ of frogs and toads, which develop their parathyroids soon after hatching, thymus feeding never produces tetanic convulsions.

A Second Mechanism to Counteract the Action of the Thymus in Salamander Larvæ.

If the parathyroids are responsible for preventing tetany in *Ambystoma opacum* and *Ambystoma maculatum*, the question may be asked why the larvæ of these salamanders are not normally victims of tetany, since they possess six well developed thymus glands and no parathyroids. It is necessary to assume that the larvæ must possess some other mechanism capable of inhibiting the action of their own thymus glands but inadequate when an excess of thymus substance is introduced into the larva.

That such a mechanism is present becomes evident from the behavior of the larvæ of a third salamander species (*Ambystoma tigrinum*) closely related to the two others. It is surprising that the larvæ of *Ambystoma tigrinum* never develop tetany even if they are fed exclusively on thymus gland of which they eat enormous quantities. *Ambystoma tigrinum* larvæ were fed from about the 14th day after hatching, on calf's thymus exclusively; some of them were kept at 25°C., others at 15°C. One of these larvæ has not metamorphosed yet, 60 weeks after hatching; it has been fed on thymus for more than a year. None of these larvæ ever showed any signs of tetany.

Yet the larvæ of the species *Ambystoma tigrinum*, like those of the two other species, do not possess parathyroids. Evidently they must have some other mechanism to counteract the tetanic effect of the thymus; and this mechanism must be sufficient not only to prevent tetany from the animal's own thymus but also tetany from an excess amount of thymus when introduced with its food.

CONCLUSIONS.

From the facts stated in this paper it is evident that the thymus gland of mammals contains a substance which is capable of producing

tetany when fed to the larvæ of certain species of salamanders (*Ambystoma opacum* and *Ambystoma maculatum*). As long as the larvæ have not developed their own thymus glands, they are able, by means of some mechanism, to counterbalance the tetanic action of the thymus substance introduced in their food. When, however, the secretion from their own thymus glands is added to the thymus material introduced with the food, this mechanism of preventing tetany becomes inadequate and tetany ensues. In the larva of a third species of salamander, *Ambystoma tigrinum*, this mechanism will prevent tetany even when the larvæ are fed on thymus.

In mammals the parathyroids are known to prevent tetany and are supposed either to absorb the tetany-producing substance and thus prevent its action or to change it into another non-toxic substance. It is at least probable that in the amphibians the parathyroids play the same rôle. Larvæ of anuran amphibians, which develop their parathyroids soon after hatching, never show tetanic convulsions if they are fed on thymus, but in certain species of salamanders, whose parathyroids develop only during metamorphosis, the larvæ invariably have tetanic convulsions upon thymus feeding, while the metamorphosed animals never show tetany.

But in addition to the parathyroids the salamanders must possess still another mechanism which during the larval period inhibits the production of tetany by the animal's own thymus glands. In the larvæ of *Ambystoma opacum* and *Ambystoma maculatum* this mechanism is sufficient only to prevent tetany from the animal's own thymus, while in the larvæ of *Ambystoma tigrinum* it is capable of preventing tetany even when the larvæ are fed with thymus.

If the thymus is the organ by whose action tetany is produced, we can understand why tetany in human beings occurs far more frequently in children than in adults, since in the latter the thymus gland is replaced, at least to a great extent, by connective tissue. The relation of thymus to tetany may also possibly explain the occurrence of tetany during pregnancy; while the parathyroids of the mother may be sufficient to prevent tetany from her largely atrophied thymus, they may not be sufficient to prevent tetany from the excess of thymus substance furnished by the fetus to the blood of the mother.

BIBLIOGRAPHY.

Baldwin, F. M., Pharyngeal derivatives of *Amblystoma*, *J. Morphol.*, 1917–18, xxx, 605.

Biedl, A., Innere Sekretion, Berlin, 2nd edition, 1913.

Dustin, A. P., Recherches d'histologie normale et expérimentale sur le thymus des Amphibiens Anoures, *Arch. biol.*, 1913, xxviii, 1.

Maximow, A., Untersuchungen über Blut und Bindegewebe. IV: Ueber die Histogenese der Thymus bei Amphibien, *Arch. mikr. Anat., Ite Abt.*, 1912, lxxix, 560.

Stejneger, L. H., and Barbour, T., A check list of North American amphibians and reptiles, Cambridge, 1917.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, September 20, 1918, Vol. i, No. 1, pp. 33–36.]

FURTHER PROOF OF THE EXISTENCE OF A SPECIFIC TETANY-PRODUCING SUBSTANCE IN THE THYMUS GLAND.

BY EDUARD UHLENHUTH.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 8, 1918.)

In the preceding note[1] it has been shown that salamander larvæ which are fed exclusively on calf's thymus gland develop tetanic convulsions. Several facts were mentioned supporting the idea that this phenomenon is identical with the tetany observed in mammals after parathyroidectomy; it was also made probable by our experiments that the tetanic convulsions, produced in the salamander larvæ, were due to the action of a specific substance contained in the thymus, inasmuch as the thymus feeding did not become effective until the primordial thymus glands of the larvæ had reached a stage in which they assume some of the structures of true thymus glands.

Instead of assuming that the thymus gland secretes a toxin causing tetany, it might be argued that the effects of thymus feeding were due to the absence from the thymus of some substances which are necessary to make up a complete diet for the salamander larvæ, for in the experiments reported in our previous paper the larvæ were fed exclusively on thymus. In other words, it might be argued that the tetany produced in our experiments was in reality a deficiency phenomenon.

While formerly it was generally assumed that the thymus belongs to the glands with internal secretion, doubts have recently arisen in this regard. After it had apparently been demonstrated by some authors that the thymus contains substances which stimulate the growth of tadpoles of frogs and toads[2] in a specific way, Swingle[3] has

[1] Uhlenhuth, E., *J. Gen. Physiol.*, 1918, i, 23.

[2] Gudernatsch, J. F., *Arch. Entwcklngsmechn. Organ.*, 1913, xxxv, 457; *Am. J. Anat.*, 1913–14, xv, 431.

[3] Swingle, W. W., *J. Exp. Zool.*, 1917–18, xxiv, 521.

recently published feeding experiments performed on tadpoles of frogs which seem to prove that the feeding of such larvæ with thymus gland of mammals has no influence whatever on growth. The writer's[4] own experiments on salamanders demonstrate clearly that during the larval period of these animals, thymus when given as food may considerably change the rate of growth, but that this is not due to a specific influence of that gland upon growth.

It is, therefore, of interest that we are able to show that the tetany produced in thymus-fed salamander larvæ is not due to a deficiency in the thymus of one or several substances, but is due to the presence in the thymus of a specific tetany-producing substance.

EXPERIMENTAL.

If tetany in salamander larvæ fed on thymus is due to the absence from the thymus of one or more substances, it is clear that the addition of a sufficient amount of normal food to the thymus diet should prevent the development of tetanic convulsions. It was found, however, that this is not the case.

Thirty larvæ of the species *Ambystoma maculatum*—from the same mother and of the same age—were fed on thymus from about the 5th week after hatching. The larvæ developed tetany very soon; convulsions were noticed as early as 15 days after the first thymus meal. At the end of the 3rd week, the series was divided into two lots, each containing fifteen individuals. One group was fed on thymus exclusively, the other one was fed alternately one day on thymus and one day on earthworms, the latter constituting a complete diet in the case of the salamanders.

The result of this experiment was that tetany is not stopped by the addition of a sufficient amount of complete diet, but on the contrary developed still further towards a maximum. Therefore, the tetanic convulsions resulting from thymus feeding are not due to a deficiency in the diet but must be due to a specific tetany-producing substance contained in the thymus gland.

It was also noticed that in the mixed food series the tetany period was shorter than in the thymus series and that the percentage of ani-

[4] Uhlenhuth, *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 37.

mals suffering from tetany was smaller on a mixed diet than on pure thymus diet. This is due to several factors.

First, it was pointed out in a previous article[1] that the salamander larvæ, though they have no parathyroids, must possess some other mechanism capable of doing away with a certain limited amount of the tetany-producing poison; otherwise even normal larvæ not fed on thymus should show tetany as soon as their own thymus glands are sufficiently developed. In one species (*Ambystoma tigrinum*) this mechanism is strong enough to prevent tetany even if by means of feeding thymus exclusively the body is flooded with a large amount of the tetany toxin. Hence, it is not surprising that if a smaller dose of the toxin is introduced into the body, the larvæ should be capable of removing—or antagonizing—so much of the toxin that the remaining part may be less effective than a maximum dose would be. Hence the fact that tetanic attacks occurred less frequently in each single individual on a mixed diet than on an exclusive thymus diet is due to the difference in the amount of thymus substance.

A second factor is still more important as regards the decrease of tetany in the mixed food series than the first factor mentioned. It has been pointed out in another communication[1] that larvæ of the species of *Ambystoma maculatum* and *Ambystoma opacum* cease to show tetanic convulsions after metamorphosis, probably because of the action of the parathyroids developing during metamorphosis. Metamorphosis started in the mixed food series 7 weeks earlier than in the pure thymus series, and was finished in the former series 19 weeks earlier than in the latter series.

We have alluded to the fact that some authors believe they have demonstrated that thymus feeding retarded the metamorphosis of the tadpoles of frogs and that this effect was specific. This conclusion is not correct. It became clear in the course of our study of amphibian metamorphosis[5] that the retardation of metamorphosis upon exclusive thymus feeding is probably not due to a specific effect of the thymus, but to a deficiency in the diet. If retardation of metamorphosis upon thymus feeding were due only to the absence of certain substances from the thymus and tetany were due to the presence of certain toxic

[5] Uhlenhuth, E., *J. Exp. Zool.*, 1918, xxv, 135.

substances in the thymus, we should expect that upon addition of a complete diet to the thymus food tetany would still occur, but metamorphosis would take place in the normal way and at the normal time. In fact, metamorphosis started even a few days earlier than in a control series consisting of thirty larvæ of the same age. Thus by adding normal food to the thymus diet the specific actions of the thymus may be sifted out from the non-specific ones.

The tetany produced by exclusive thymus feeding is a specific effect of the thymus due to the existence of a tetany-producing poison in that gland, while the retardation of metamorphosis in the case of an exclusive diet of thymus is due to a deficiency in the diet.

CONCLUSIONS AND SUMMARY.

The effect of the thymus gland in producing tetany is due to a specific tetany toxin produced by and contained in the thymus, and the thymus gland must be added to the group of glands for which the function of internal secretion has been demonstrated.

[Reprinted from The Journal of Bacteriology, September, 1918, Vol. iii, No. 5, pp. 437–440.]

THE ENDO MEDIUM FOR THE ISOLATION OF B. DYSENTERIAE AND A DOUBLE SUGAR MEDIUM FOR THE DIFFERENTIATION OF B. DYSENTERIAE, SHIGA AND FLEXNER.[1]

By I. J. KLIGLER and J. DEFANDORF.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 24, 1917.)

This paper is based on a study of means of isolating *Bacillus dysenteriae* from human excreta and polluted soil or water, in which it may be contained. Previous experience (Kligler, 1918) had shown that with respect to sensitiveness to chemicals, the dysenteric group of bacilli approached the Gram positive bacteria; or, in other words, departed from the typhoid and paratyphoid groups. On the other hand, when an enriching medium was employed for cultivation (nutrose, bile, egg), it proved just as favorable to the growth of other intestinal bacteria as to *B. dysenteriae*. Hence resort was had to a plate medium that would afford colony differentiation and permit at the same time the development of very delicate strains of the dysentery bacillus. Of the two available media for this purpose, litmus-lactose agar and Endo's medium, the latter presented certain advantages.

Modified Endo Medium.—In carrying forward the cultivation on the Endo medium it was discovered that pure cultures of the Shiga dysentery bacillus or cultures in fecal emulsions sometimes gave irregular results. It appeared at first that the fuchsin acted as the inhibitive agent, being more effective against the Shiga bacillus than against the Flexner group of bacilli. In following out this line of experiment, it was determined that the addition either of nutrose or bile produced a marked improvement of the medium, the former

[1] Work aided by a grant from the International Health Board of The Rockefeller Foundation.

surpassing the latter in this respect. Further study showed, however, that other factors were concerned, and finally, by elimination and new cultivation, a medium was evolved which offered distinct advantages for the cultivation of the dysentery bacilli. The modification consists in the substitution of sodium bisulphite as recommended by Robinson and Rettger (1916) and the accurate adjustment of the hydrogen ion concentration to a P_H value of 7.6–7.8. The medium as ordinarily prepared with a phenolphthalein reaction of +0.2 gives an end reaction, after the addition of the sodium-sulphite which is alkaline, ranging from P_H 8.4 to 8.8. This degree of alkalinity is inhibitive to the growth of the Shiga bacillus.

Either meat-infusion or beef-extract agar may be used. We use a beef-extract medium prepared as follows:

Peptone	10.00 grams
Beef extract	3.00 grams
NaCl	5.00 grams
Agar	15.00 grams
Water	1000 cc.

All the ingredients except the agar are dissolved first; the agar is then added and the mixture autoclaved for one hour at 15 pounds pressure. It is then cooled to 50°C., white of egg added (2 eggs to 5 liters), and steamed in the Arnold for thirty minutes. The reaction is then adjusted to P_H 7.4, with phenolsulphonephthalein as the indicator, the medium boiled on the free flame for six to seven minutes, filtered, flasked, and autoclaved.

This constitutes the stock[2] medium from which all the special media—brilliant green, Endo, etc.—may be prepared. Before pouring the Endo plates the reaction is adjusted to P_H 7.6–7.8, and the lactose and the fuchsin-sulphite are added in the usual manner. It has been our experience that about 0.8 cc. N NaOH was required for every 100 cc. of agar. It is necessary, however, to determine the exact amount for each fresh lot of agar.

[2] This agar can be used for all purposes. The reaction is favorable for the growth of all common bacteria. In places where a large amount of medium is used, and in the field, it is especially advantageous to have a single stock that can serve as a basis for the various modified media.

Double Sugar Medium.—Having developed the colonies on the Endo plate, it is necessary to carry the differentiation further so as to confirm the nature of the selected colonies. Russell (1911) makes use of a double sugar agar containing 0.1 per cent glucose and 1 per cent lactose. While this medium is effective, it does not permit the distinction of *B. typhosus* and the two classes—alkaline or Shiga and acid or Flexner, etc.—of dysentery bacilli from each other. We have substituted 0.5 per cent mannite for the lactose, with the effect of accomplishing the purpose.

The selected colonies are stabbed in the butt of the slanted tube and also streaked on the surface. Andrade's indicator is used. The differentiation is made after twenty-four hours' incubation at 37°C. The Shiga bacillus yields a slight reddening of the butt but no change of the surface. The acid class of dysentery bacilli and *B. typhosus* color the entire medium red; no gas is produced. *B. alcaligenes* produces no changes; while the paratyphoid bacilli cause both reddening and gas formation. *B. proteus*, on the other hand, produces gas but no reddening. If decolorized fuchsin is employed as indicator, then the paratyphoid bacilli A and B can be distinguished from each other, as the latter brings about final decolorization of the medium. However, the main advantage of this medium is that it permits of a more rapid separation of the two classes of dysenteric bacilli—the Shiga and the Flexner.

SUMMARY.

A study of the Endo medium, as applied to *B. dysenteriae*, indicated that the most important single condition that must be carefully controlled, particularly if the medium is intended for the isolation of dysenteric bacilli, is the end reaction. The ingredients are of significance in so far as they furnish the elements essential for the growth of the organisms. Given an otherwise favorable Endo medium, however, the Shiga bacillus will or will not grow, depending on whether the end reaction is P_H 7.6–7.8 or P_H 8.4–8.8, the reaction ordinarily obtained.

A mannite-glucose double sugar medium on the principle of Russell's double sugar agar is described, to be employed for rapid differentiation of the Shiga and Flexner class of dysenteric bacilli.

REFERENCES.

KLIGLER, I. J. 1918 J. Exp. Med., **27**, 463.
RUSSELL, F. F. 1911 J. Med. Res., **25**, 217.
ROBINSON, H. C., AND RETTGER, L. F. 1916 J. Med. Res., **34**, 363.

[Reprinted from THE JOURNAL OF BACTERIOLOGY, September, 1918, Vol. iii, No. 5, pp. 441–444.]

NOTE ON CROSS-AGGLUTINATION OF B. COLI COMMUNIS AND B. DYSENTERIÆ SHIGA.[1]

BY I. J. KLIGLER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 24, 1917.)

This note refers to an instance of cross-agglutination between two distinct bacilli in immune sera, prepared for each, and in practically identical end dilutions.

Other similar instances of cross-agglutinations are reported in the literature, but with few exceptions either the agglutination limit was less for the heterologous than for the homologous organism, or the coöperation of the two organisms in producing the agglutinins could not be excluded. Thus Stern (1898) studied five samples of blood serum from patients with typhoid fever which agglutinated, equally with the typhoid bacilli, the cultures of *B. coli* isolated from their stools. In this example, the possibility exists that mixed infection with the colon bacilli coexisted. Rodet (1897) found that sheep sera derived from animals immunized with *B. typhosus* and *B. coli*, respectively, agglutinated the heterologous organism equally with the homologous. Park and Williams (1910) observed the serum of a horse immunized with the Flexner dysenteric bacilli which agglutinated a culture of *B. coli* in the same end dilutions (1: 10,000) as the dysenteric bacillus. Conversely, a goat immunized with the *B. coli* culture yielded a serum of a titre of 1: 5,000 for the *B. coli* and 1: 3,000 for Flexner *B. dysenteriæ*. The chief interest of the following communication arises from the fact that it concerns a similar cross-agglutination of *B. coli* and *B. dysenteriæ* Shiga.

EXPERIMENTAL.

The culture of the *B. coli* used in the tests was recently obtained from the stool of a chronic carrier of *B. typhosus*. On inoculation

[1] Work conducted under a grant of the International Health Board of The Rockefeller Foundation.

into rabbits it proved pathogenic, but never induced the paralysis characteristic of the Shiga bacillus inoculation of that animal. The Shiga bacillus had been cultivated outside the body for a long time; the original culture was obtained from Dr. Wadsworth of the New York State Department of Health. It fulfilled all the morphological and cultural requirements and upon inoculation into rabbits induced

TABLE 1.

Particular and General Agglutination Tests with Special B. coli and B. dysenteriæ Sera.

CULTURE		DYSENTERY SERUM						COLON SERUM					
		100	500	1000	2000	4000	C	100	500	1000	2000	4000	C
B. dysenteriæ (Shiga)	30	++	++	++	+	±	−	++	++	++	++	+	−
	6	++	−	−	−	−	−	+	−	−	−	−	−
	27	−	−	−	−	−	−	−	−	−	−	−	−
B. coli	13	++	++	++	+	±	±	++	++	++	++	+	±
	11	−	−	−	−	−	−	±	−	−	−	−	−
	12	−	−	−	−	−	−	−	−	−	−	−	−
	14	−	−	−	−	−	−	−	−	−	−	−	−
	95	−	−	−	−	−	−	−	−	−	−	−	−
	104	−	−	−	−	−	−	−	−	−	−	−	−
	137	−	−	−	−	−	−	−	−	−	−	−	−
B. dysenteriæ (Flexner)	2	−	−	−	−	−	−	±	−	−	−	−	−
	22	−	−	−	−	−	−	−	−	−	−	−	−
	23	−	−	−	−	−	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−	−	−	−	−	−
	25	−	−	−	−	−	−	−	−	−	−	−	−
	26	±	−	−	−	−	−	−	−	−	−	−	−
	28	−	−	−	−	−	−	−	−	−	−	−	−
	29	−	−	−	−	−	−	−	−	−	−	−	−
	S	−	−	−	−	−	−	−	−	−	−	−	−

the typical paralysis. The two organisms had the property in common of tending to spontaneous agglutination.

The property of reciprocal agglutination was limited to the two strains described. Other cultures of *B. coli* and *B. dysenteriæ* Shiga did not exhibit it. That is, agglutinating sera prepared with the particular strains of the colon and Shiga dysentery bacilli men-

tioned reacted on each other quite as on their own cultures, while other colon bacilli showed no especial agglutinating capacity with the Shiga immune serum nor did other Shiga bacilli, with the colon agglutinating serum. Similarly the stock polyvalent dysenteric horse serum agglutinated the particular *B. coli* strain at 1 : 2,000, which was its value to its homologous serum.

TABLE 2.

Absorption Tests for Agglutinins Performed with Homologous and Heterologous Cultures.

SERUM	ABSORBING CULTURE	AGGLUTINATION TEST CULTURE	DILUTIONS						
			250	500	1000	2000	4000	8000	C
30	13	13	−	−	−	−	−	−	−
		30	++	++	+	±	±	−	−
	30	13	±	−	−	−	−	−	−
		30	+	−	−	−	−	−	−
13	13	13	±	−	−	−	−	−	−
		30	+	−	−	−	−	−	−
	30	13	++	++	++	++	++	−	−
		30	++	+	−	−	−	−	−
Controls.... 30	None	13	++	++	++	+	−	−	−
		30	++	++	++	++	−	−	−
13	None	13	++	++	++	++	++	−	−
		30	++	++	++	++	+	±	−

Single colonies of each organism were selected after several platings and employed to immunize rabbits. The injections were given at three day intervals. The rabbit receiving the culture of *B. coli* was given five intravenous injections, beginning with 1/100 and ending with 1/10 of an agar slant of living culture. The rabbit receiving the culture of the Shiga bacillus received seven intravenous injections, beginning with 1/50th of a slant of a killed culture and ending with 1/10th of the living culture.

The results with the sera prepared from isolated colonies of each bacillus coincided with those of the original test. The fact should be mentioned that the particular Shiga immune serum was without agglutinating action on other Shiga cultures or cultures of the Flexner dysenteric group. Table 1 brings out in brief form the points just described. The cross-agglutinating colon bacillus culture is number 13 and the Shiga bacillus culture number 30.

Absorption of the immune sera was carried out with the homologous and heterologous agglutinating cultures, after which agglutination tests were made. The results are given briefly in table 2.

SUMMARY.

Two cultures are described, one a typical *B. coli* and the other a typical *B. dysenteriæ* Shiga, culture, which yield immune sera possessing agglutinating properties of practically equal quantity for each culture. Absorption experiments made with each culture upon each kind of immune serum indicate that two distinct agglutinins are yielded in about equal amount in the process of immunization of rabbits with the respective cultures. The two agglutinins are specific ones, each for its own culture, and accessory (paragglutinin), each for the other culture. The absorption of the accessory agglutinin leaves the specific agglutinin quantitatively unaffected.

The significance of this reciprocal agglutinative property in respect to the two bacilli described can only be surmised. The obvious suggestion is a group relationship between certain strains of colon and dysenteric bacilli, a subject hardly to be pursued profitably in this connection.

REFERENCES.

Castellani, A. 1902 Ztschr. f. Hyg., **40**, 1.
Park, W. H., and Williams, W. 1910 Pathogenic Microorganisms, 166, 168.
Rodet, A. 1897 Compt. rend. de la Soc. de Biol., **4**, 874.
Stern, R. 1898 Centralbl. f. Bakt., **23**, 673.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, October 1, 1918, Vol. xxviii, No. 4, pp. 377–385.]

A FURTHER STUDY OF EXPERIMENTAL PAROTITIS.

BY MARTHA WOLLSTEIN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 29.

(Received for publication, June 11, 1918.)

In a previous paper[1] it was shown that cats injected into the parotid gland and testicle with a bacterial sterile filtrate of the salivary secretion of children in the active stage of parotitis can be made to develop a pathological condition having several points of resemblance to that present in mumps in human beings.

The presence of acute cases of parotitis in military camps near New York City provided the opportunity to repeat the experiments with material from adult cases.[2]

EXPERIMENTAL.

Saliva from Patients.

In order to repeat the work done 2 years ago, mouth washings in normal saline solution were obtained from soldiers suffering from acute parotitis for 1 to 12 days. The washings were filtered through a Berkefeld candle N and the filtrate, which was sterile by ordinary aerobic and anaerobic culture methods, was inoculated into the parotid glands and testes of healthy, half grown cats.

There are several precautions to be observed in inoculating parotitis material into cats. The animals must be only half grown; old males do not react, even if they are well inoculated through the very tough

[1] Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 353.

[2] I am greatly indebted to Captain Meader for placing material at Camp Mills at my disposal, and to Captain Monaghan and Lieutenants Cowdrey and Braiton for many courtesies at the Army Hospital at Secaucus, New Jersey. Material was also obtained at Camp Jackson, Columbia, South Carolina, through the kindness of Lieutenant Colonel Roberts.

connective tissue over the parotids on the side of the face. The most marked reactions, as expressed by severe symptoms and lesions, develop when the material is inoculated within a few hours after it is obtained from the patient; that is, as soon as it can be filtered. With proper care of the filters and the glassware, contaminations do not occur; and while the filtrates must always be tested for sterility, neither time nor animals are wasted by immediate inoculation if the technique is right.

Attempts to probe Steno's duct and obtain saliva directly from the swollen parotid were unsuccessful. The opening is normally small; during an attack of parotitis the mucous membrane is red and swollen, thus reducing the size of the orifice and making it even more difficult to introduce a cannula.

Filtrates of mouth washings obtained from patients ill 1 to 3 days produced characteristic symptoms and lesions in the inoculated cats, consisting of a rise in temperature, leukocytosis with an absolute increase in mononuclear cells, and tenderness and swelling of the injected glands with definite histological changes. All the symptoms appear after an incubation period of 5 to 8 days and endure about 8 days, when they decline.

PROTOCOL I.

Cat A.—Inoculated Nov. 23, 1917, with sterile filtrate made from the combined saliva of three soldiers who had been ill 36 hours. 2 cc. injected into the right parotid.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1917	°C.		
Nov. 23	38.9	23,000	Polynuclears 60; large mononuclears 18; small mononuclears 17; eosinophils 5.
" 24	40.0	25,000	No signs.
" 26	40.3	27,000	Polynuclears 70; large mononuclears 17; small mononuclears 11; eosinophils 2.
" 27	40.5	32,000	Cat is very quiet; ill.
" 30	40.8	33,000	Polynuclears 75; large mononuclears 16; small mononuclears 19; eosinophils 0.
Dec. 3	39.6	30,000	Less ill.
" 5	39.1	35,000	Polynuclears 63; large mononuclears 12; small mononuclears 19; eosinophils 6.
" 8	38.8	38,000	Polynuclears 71; large mononuclears 16; small mononuclears 9; eosinophils 4.
" 11	38.7	40,000	Polynuclears 71; large mononuclears 13; small mononuclears 11; eosinophils 5.
" 15	39.0	35,000	Chloroformed.

Right parotid fully twice as large as the left; the adjacent lymph nodes were swollen. The parotid was edematous, pink, and granular on section contrasting with the smaller, paler, and smoother left parotid. Cultures gave no growth. Microscopically the right parotid showed edema, some degree of mononuclear cellular infiltration around the ducts, and a less degree about the blood vessels.

Material was obtained from soldiers at Camp Jackson, Columbia, South Carolina, very early in the course of a parotitis attack. It was filtered in the usual way and inoculated into two cats. Both animals reacted well. Fig. 1 illustrates the parotid, submaxillary gland, and adjacent lymph nodes from one of the cats, removed after death on the 17th day.

On the 6th day of the disease the filtered saliva produced less marked symptoms, while inoculation of material obtained from a patient 9 days or longer after onset of the mumps attack was apparently without effect. The following protocol illustrates the latter point.

PROTOCOL II.

Cat B.—Inoculated Feb. 13, 1918, with sterile filtrate made from the saliva of a soldier at Secaucus, New Jersey; onset of mumps 10 days before. 2 cc. injected into right parotid.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1918	°C.		
Feb. 13	38.9	14,000	Polynuclears 56; large mononuclears 14; small mononuclears 20; eosinophils 0.
" 14	38.7	18,000	No symptoms.
" 16	38.6	12,000	Polynuclears 72; large mononuclears 12; small mononuclears 15; eosinophils 1.
" 18	38.8	13,000	Polynuclears 75; large mononuclears 6; small mononuclears 19; eosinophils 0.
" 20	38.7	17,000	No symptoms.
" 23	39.0	20,000	Polynuclears 73; large mononuclears 8; small mononuclears 19; eosinophils 0.
" 26	39.4	22,000	Polynuclears 75; large mononuclears 11; small mononuclears 14; eosinophils 0.
" 28	39.0	23,000	Chloroformed.

The right parotid was not larger than the left, and the adjacent lymph nodes were not swollen. Microscopic examination showed no lesion in either parotid.

It will be noted that in this animal the mononuclear leukocytes were actually decreased, and the polynuclear leukocytes slightly increased, a result which had been previously noted after the injection of saliva not containing the virus of parotitis.

Cats inoculated with material from soldiers on the 9th, 11th, and 12th days of an attack of parotitis gave results similar to those described in Protocol II; that is, the temperature range was only 0.5°C., the leukocytic count was not even doubled, the mononuclear cells were decreased instead of increased, the polynuclear cells were slightly increased, and at autopsy the parotid gland showed neither macroscopic nor microscopic changes. From this we may conclude that the period of infectivity of the mouth secretions, as far as this test is capable of indicating, is comparatively short, and covers about 1 week, corresponding to the swelling of the parotid. A fresh swelling appearing in the opposite parotid gland would, of course, prolong the infectious period for a given patient.

Saliva from Cats.

The saliva from inoculated cats was employed for further injection. By anesthetizing the animals large amounts of ropy saliva are readily obtained, and at the height of the symptoms, at the end of the 2nd week after inoculation, the saliva thus obtained was found to incite symptoms and lesions in other and normal cats. The salivary secretion from normal cats obtained in the same manner and injected gave only negative results.

Following the inoculation of infected materials not only was the parotid gland affected, but the submaxillary, the sublingual, and the adjacent lymph nodes also. The extent of the swelling of the salivary glands was greater in this series of experiments than in the first series reported.[1] Moreover, the lymph nodes on the uninoculated side were observed to be swollen and moist, as though the infectious agent present in the saliva had been carried to them, presumably by lymphatics, leading to reaction on the uninoculated side. On microscopic examination the swollen lymph nodes showed necrosis of the center of several lymph nodules, while the sinuses were distended and the lining cells swollen. In this connection it may be remarked that swelling of cervical lymph nodes was a marked condition in many of the soldiers ill with mumps.

As regards the lesions in the parotid glands and testes, it may be stated that they agreed with the descriptions given in the previous paper[1] and briefly are as follows: In the parotid there are edema of the interlobular connective tissue and mononuclear cell infiltration about the ducts, and occasionally about the blood vessels as well. Similar lesions have been described in the pathology of cases of human mumps. In the testis there is principally an epithelial degeneration resulting in diminished spermatogenesis.

Blood.

The marked constitutional symptoms observed in many patients with mumps suggested an investigation of the blood at the height of the attack.

Films were stained with Wright's and with Giemsa's stain. Cultures were made in the usual way and by the anaerobic (tissue ascitic

broth) method of Noguchi.[3] No parasites were detected under the microscope or cultures obtained. Blood was taken from a vein at the elbow in seven patients ill 2 to 3 days who were suffering from marked constitutional symptoms. The blood was defibrinated and had been proved sterile in glucose broth and on agar plates; it was inoculated into normal cats. The protocol of such an experiment is given, together with its control.

PROTOCOL III.

Cat C.—Inoculated Mar. 13, 1918, with defibrinated blood taken from the arm vein of a patient ill 3 days. 2 cc. injected each into right testis and right parotid.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1918	°C.		
Mar. 13	39.0	13,000	Polynuclears 67; large mononuclears 28; small mononuclears 5; eosinophils 0.
" 14	39.2	34,000	Polynuclears 80; large mononuclears 9; small mononuclears 11; eosinophils 0.
" 15	40.4	47,000	No signs.
" 18	39.2	35,000	Polynuclears 74; large mononuclears 20; small mononuclears 5; eosinophils 0. Right testis tender.
" 22	39.3	40,000	Testis tender.
" 26	39.5	43,000	Polynuclears 84; large mononuclears 4; small mononuclears 11; eosinophils 1.
" 28	38.8	40,000	Seems quiet and ill. Polynuclears 82; large mononuclears 6; small mononuclears 12.
" 30	39.0	38,000	Chloroformed.

Right parotid, submaxillary, and lymph nodes are swollen and pink and larger than the left (Fig. 2). Right testis also swollen. Microscopically the right parotid gland showed marked edema and swelling of the epithelial cells in some acini. The lymph nodes showed distention of the sinuses and swelling of the lining cells. The right testis showed a degree of characteristic epithelial degeneration.

[3] Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

PROTOCOL IV.

Cat D.—Inoculated Mar. 19, 1918, with sterile defibrinated blood from a healthy adult man. 2 cc. injected each into the right parotid and right testis.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1918	°C.		
Mar. 19	39.1	19,000	Polynuclears 60; large mononuclears 22; small mononuclears 18; eosinophils 0.
" 20	38.9	26,000	Polynuclears 72; large mononuclears 24; small mononuclears 4; eosinophils 0.
" 23	39.2	20,000	Animal well.
" 25	39.5	20,000	Polynuclears 72; large mononuclears 16; small mononuclears 12; eosinophils 0.
" 28	39.0	25,000	No signs.
Apr. 1	38.9	25,000	Polynuclears 65; large mononuclears 10; small mononuclears 25; eosinophils 0.
" 4	39.1	23,000	Chloroformed.

Both parotid glands of equal size; slight subcapsular erythrocytic discoloration in the right. No difference in the two testes.

An examination of Protocol III shows that while the temperature range was small it was somewhat greater than in Protocol IV; the blood count, on the other hand, showed a polynuclear leukocytosis in both animals, but while the mononuclear cells were diminished in the control cat they were actually increased throughout in the animal receiving the blood from the patient ill of parotitis. Identical results were obtained with the blood of four other patients which had been pooled before inoculation. The blood taken from a case with slight symptoms on the 5th day of illness gave no result on injection into a cat.

The conclusion drawn from this experiment is to the effect that the blood of parotitis patients, especially those suffering from severe constitutional symptoms, is infective for cats. This conclusion is in keeping with the clinical course and metastatic complications of certain cases. It may be remarked that one of the four patients whose blood was pooled suffered from orchitis. Meningitis has not infrequently been described, especially in the French literature, as complicating mumps. In one suspicious case among our series of patients, the spinal fluid was clear, contained no lymphocytes, and was without effect when inoculated into cats.

Recurrent Parotitis.

An opportunity was afforded of studying a case of so called recurrent mumps. The patient, a soldier, had a first attack in November, 1916. He came under my observation in March when he was suffering either from another attack or merely from an exacerbation, as the swelling had persisted somewhat since November. Cases of parotitis which recur after 3 or 4 weeks are not uncommon, and Jochmann[4] states that the swelling may endure for months or even for a year. In this patient the right parotid remained enlarged longer than the left; both had returned to about normal size in 6 months. Three sets of inoculations were made with the salivary secretion: one on March 13, one on May 1, and the last on May 15. The first two gave positive, the last a negative result. As regards the last it may be stated that the swelling of the parotid had disappeared soon after May 1. Fig. 3 shows the condition found in the glands after the second inoculation.

SUMMARY.

A new series of inoculations into cats of the filtered sterile salivary secretions derived from cases of parotitis has been described. They confirm the observations made in 1915–1916 and extend them to include the epidemic parotitis occurring in our military forces.

Incidentally confirmatory evidence of the filterable nature of the causative agent of mumps has been obtained.

It has been determined that the saliva of man and of inoculated cats, and the inoculated glands of the latter animals, contain the filterable, infective agent.

The lesions present in the inoculated organs conform to those described in our first publication. In addition, the lymph glands adjacent to the salivary glands on the uninoculated side were sometimes found to be swollen and to exhibit microscopic lesions. Probably the involvement resulted from salivary and lymphatic infection.

The "virus" of parotitis was detected most readily in the saliva during the first 3 days of the disease, less easily on the 6th day, and not

[4] Jochmann, G., Lehrbuch der Infektionskrankheiten für Aerzte und Studierende, Berlin, 1914.

at all on the 9th day. It was detected also in the blood of patients showing marked constitutional symptoms, and in the saliva of a case of recurrent mumps at the periods of enlargement of the parotid glands, but not 2 weeks after the swelling had subsided. It was not detected in the cerebrospinal fluid.

EXPLANATION OF PLATE 29.

FIG. 1. Parotid (*P*) and submaxillary (*SM*) glands with adjacent lymph nodes (*L*), showing swelling of the inoculated right side. Cat killed after 17 days.

FIG. 2. Parotid (*P*), submaxillary (*SM*), sublingual glands, and adjacent lymph nodes (*L*), showing swelling of the right side inoculated with blood from a patient ill 3 days. Cat killed after 18 days.

FIG. 3. Parotid (*P*), submaxillary (*SM*), sublingual (*SL*) glands, and adjacent lymph nodes (*L*), showing swelling of the right side inoculated with saliva filtrate from a recurrent case (second recurrence). Cat killed after 15 days.

FIG. 1.

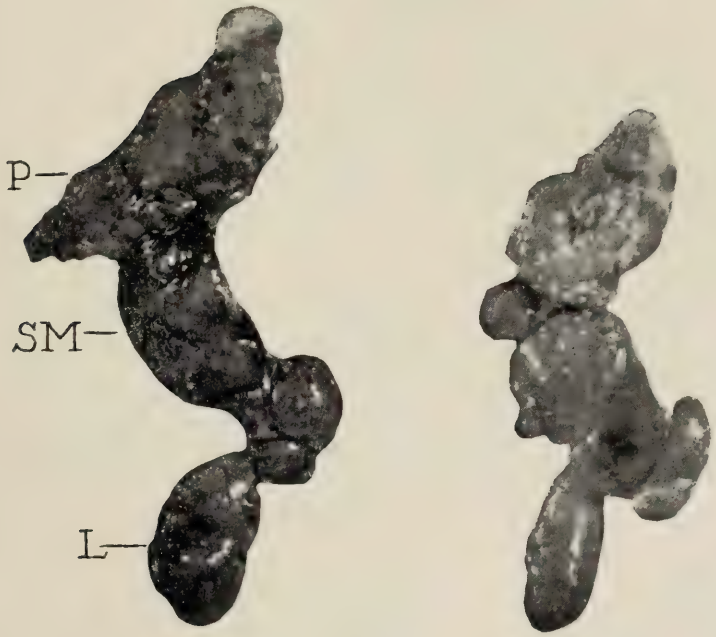


FIG. 2.

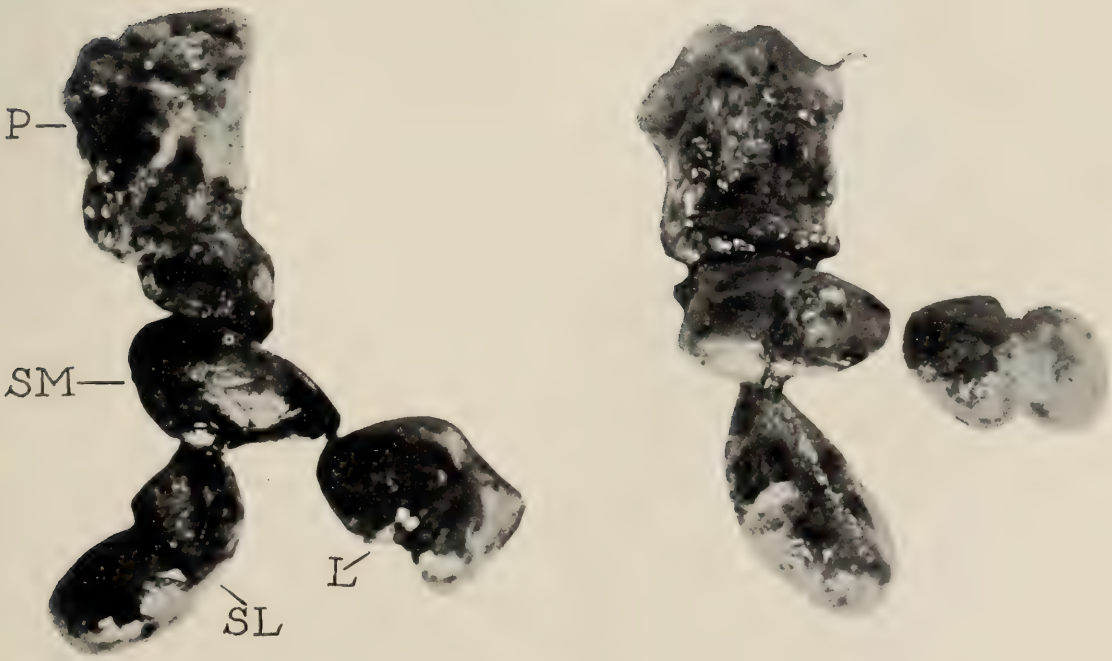


FIG. 3.

(Wollstein: Experimental parotitis.)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, October 1, 1918, Vol. xxviii, No. 4, pp. 429–434.]

THE RAT AND POLIOMYELITIS: AN EXPERIMENTAL STUDY.

BY HAROLD L. AMOSS, M.D., AND PETER HASELBAUER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 1, 1918.)

Under the title of "The rat and infantile paralysis: a theory," Richardson[1] has brought together such hypothetical data in favor of the rat and its parasite, the flea, as the active agents in the transmission of poliomyelitis as he could assemble. He has also brought these evidences to bear on the epidemiology of the disease and has concluded that:

"In the transfer of the infection from the rat to man, the agency of the flea is assumed, although the possible contamination of food by rodent excretions might well be considered. The insect transfer might well be simply mechanical or it might require a preliminary cycle of development of the virus in the flea..... The foregoing theoretical considerations have been put forward as explaining better than any other hypothesis as yet submitted, the epidemiological facts as observed in infantile paralysis."

Richardson further states that extended experimental investigation will be necessary to determine the validity of his theory.

Various kinds of animals have from time to time been incriminated in the spread of epidemic poliomyelitis. Perhaps the one most often cited is poultry because of the paralytic disease, limber-neck, to which they are subject. Doty[2] in his epidemiological report of the New York epidemic of 1916 gives consideration to and excludes from account the rat as an agent of transmission of the disease, and he quotes Professor L. O. Howard to the effect that no marked epizootics among rats have been noted in connection with the larger epidemics of infantile paralysis.

Along with the failure to discover a reservoir of the causative microorganism outside man, by inoculating the nervous tissues from animals suspected to have suffered from poliomyelitis into monkeys,

[1] Richardson, M. W., *Boston Med. and Surg. J.*, 1916, clxxv, 397.

[2] Doty, A. H., Special investigation of poliomyelitis, 1916, Department of Health, City of New York, 40.

unsuccessful attempts have been made to induce paralysis in various kinds of animals, including the white rat, by direct injection of infected nervous tissues into the brain.[3] It is, however, not evident that any systematic effort has been made to detect the virus of poliomyelitis in the rat or to discover whether, when experimentally injected, it is capable of surviving in that animal. Richardson refers in a parenthesis to a statement by Rosenau[4] that while he has apparently been able to produce paralytic disease in rats by inoculation of the virus, yet the experimental data are not sufficiently advanced to justify conclusions. As no confirmation of this important point has been offered in the 2 intervening years, we may assume that convincing evidence has not been obtained.

EXPERIMENTAL.

Our experiments have taken two directions. In the first place, we undertook to transmit poliomyelitis to monkeys by employing for inoculation the central nervous organs of wild rats caught in the localities in Brooklyn in which numerous cases of the epidemic disease prevailed in the summer of 1916. Through the cooperation of the Deputy Commissioner of Health, Dr. John S. Billings, we secured a considerable number of rats. The animals, trapped in cages, were chloroformed and the central nervous and other organs carefully removed and preserved in 50 per cent glycerol in the refrigerator. In the next place, we injected active poliomyelitic virus (suspensions of brain and spinal cord of paralyzed monkeys) into the brain of white rats. As pressure effects which may cause death in a few hours are readily produced, several rats are injected at once. The purpose was to determine whether the virus is capable of surviving in the brain of the rat for any length of time even though poliomyelitis is not induced. Should the virus be quickly destroyed, the conclusion would be justified that the rat organism is not favorable to the virus, from which the further deduction, that this animal improbably serves as a reservoir of the virus in nature, might be made.

[3] Flexner, S., *J. Am. Med. Assn.*, 1910, lv, 1105.

[4] Rosenau, M. J., cited from Richardson,[1] p. 400.

Series I. Inoculation of Organs of Rats into Monkeys.

Experiment 1.—April 11, 1917. 10 per cent suspension was made of the brain and spinal cord of Rats 1, 2, and 3 collected in Brooklyn. 2 cc. of the suspension were injected intracerebrally and 15 cc. intraperitoneally into a *Macacus rhesus*. April 25. No symptoms having appeared, the inoculations were repeated in the same manner and with a 10 per cent suspension prepared from the nervous organs of the same rats. No symptoms whatever developed.

Experiment 2.—April 11, 1917. 10 per cent suspension was made of the brain and spinal cord of Rats 4, 5, and 6 obtained through Dr. Billings. 2 cc. were injected intracerebrally and 15 cc. intraperitoneally into a *Macacus rhesus*. April 25. No symptoms having developed, the injections were repeated with a 10 per cent suspension prepared in the same manner from the nervous organs of the same rats. The animal never showed symptoms.

Experiment 3.—April 11, 1917. The spleen, liver, and kidney of Rats 1, 2, and 3 were made into a 10 per cent suspension. Of this, 2 cc. were injected intracerebrally and 12 cc. intraperitoneally into a *Macacus rhesus*. April 25. No symptoms having appeared, the inoculations were repeated in the same manner and with a 10 per cent suspension prepared from the same viscera. No symptoms developed subsequently.

The significance of this experiment is obvious. Materials from six rats collected in Brooklyn in the vicinity in which the epidemic of poliomyelitis was severe, were injected into three *Macacus rhesus* monkeys under conditions sufficing to incite infection in these animals provided the poliomyelitic virus had been present in the internal organs in any considerable amount and of any real virulence. Moreover, not only did the monkeys fail to respond to a single inoculation of large quantities of the organs of the rats, but they failed equally to react to a second injection of this material made 2 weeks later. It may therefore be concluded that none of the six rats tested carried demonstrable amounts of the poliomyelitic virus.

The next tests are a repetition of those carried out by Amoss[5] on the power of survival of an active virus of poliomyelitis when injected into the brain of rabbits. The tests had been made in the course of a critical examination of the contentions of Rosenow[6] and

[5] Amoss, H. L., *J. Exp. Med.*, 1918, xxvii, 443.

[6] Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxvii, 1202; *Science*, 1916, xliv, 614; *J. Am. Med. Assn.*, 1917, lxviii, 280.

his coworkers and Nuzum[7] and his associates that poliomyelitis may be induced in rabbits by means of streptococci. Amoss[5] found that the virus of poliomyelitis when injected into the brain of rabbits did not survive there as long as 7 days. The rabbit was therefore regarded as unfavorable to the mere presence within its body of the true virus of poliomyelitis although permitting, on the contrary, streptococci both to survive and multiply in its organs.

Series II. Survival of the Virus in Rats.

Experiment 4.—Under ether anesthesia 0.15 cc. of a suspension of equal parts of active glycerolated monkey virus (spinal cord) and isotonic salt solution was injected intracerebrally into each of four white rats. Two died from pressure within 24 hours. The remaining two were observed for 7 and 14 days respectively and then etherized. Neither developed any symptoms, and on autopsy showed any lesions at the site of inoculation. In each instance the brain tissue surrounding the site of inoculation was made into a suspension with equal parts of isotonic salt solution and injected intracerebrally into a *Macacus rhesus* monkey. No symptoms of poliomyelitis developed.

Experiment 5.—Five white rats were given intracerebral injections of 0.1 cc. of a 50 per cent suspension of active virus in the manner described in the previous experiment. Four succumbed to pressure, and only one survived. The rat which survived and which had shown no symptoms was etherized on the 4th day and the excised site of inoculation in the brain suspended in 2 cc. of isotonic saline solution and injected intracerebrally into a *Macacus rhesus*. The monkey remained well. Because of the brief period of time elapsing between the inoculation of the rat and of the monkey, this experiment was repeated, with the same result.

The tests thus far conducted indicated that the virus of poliomyelitis was incapable of surviving as long as 4 days in the brain of the rat. The question arose whether this result might not be accounted for not by the rapid destruction of the injected virus, but by the small amount which was introduced; in other words, whether the minimal quantities of virus, capable of being injected into the brain without causing pressure effects, were not below the infecting dose when transferred with the rat's brain tissue to the monkey. As a matter of fact 0.1 to 0.15 cc. of the 50 per cent suspension of the spinal cord contains from 10 to 50 minimal lethal doses of the virus for monkeys

[7] Nuzum, J. W., and Herzog, M., *J. Am. Med. Assn.*, 1916, lxvii, 1205. Nuzum, J. W., *ibid.*, 1916, lxvii, 1437; 1917, lxviii, 24.

according to its initial potency. However, the next experiment was designed to answer this question and it showed that the failure to infect is not due to subminimal doses of virus introduced into the rat.

Experiment 6.—A white rat was given an intracerebral injection of 0.1 cc. of a 50 per cent suspension of the virus. 1½ hours later, and after the recovery from ether, the animal was killed with ether and the brain removed. The site of inoculation was excised, suspended in isotonic salt solution, and injected intracerebrally into a *Macacus rhesus*, of which the following is the history:

May 20, 1918. Received intracerebrally 1 cc. of the suspension described. May 26. Somewhat excited. May 27. Tremor, ataxia, weakness of left and complete paralysis of right arm. May 28. Prostrate, etherized.

The autopsy showed typical lesions of poliomyelitis confirmed by microscopic examination of the medulla, spinal cord, and intervertebral ganglia.

DISCUSSION.

The above experiments bring out a remarkable similarity between the tests on the rat and those made by Amoss[5] on the rabbit. Indeed, it now appears that the rat is an even more unfavorable host for the virus than is the rabbit, since in the former animal an effective dose of the virus for the monkey was no longer detectable at the expiration of 4 days. This fact is hardly consistent with the theory that the rat constitutes a natural reservoir in nature of the virus of poliomyelitis. Rather the experiments would indicate that the rat's organism is wholly unadapted to its multiplication and survival.

CONCLUSIONS.

The central nervous organs and other viscera of six rats, collected in a district in Greater New York in which many cases of epidemic poliomyelitis occurred, have been proved incapable of inciting, on inoculation, experimental poliomyelitis in *Macacus rhesus* monkeys.

The virus of poliomyelitis injected into the brain of white rats does not survive there as long as 4 days in a form or in amounts sufficient to cause infection when inoculated intracerebrally into monkeys.

The failure of the virus injected into the brain of rats to incite infection in monkeys is not due to the quantity introduced, since at the

expiration of $1\frac{1}{2}$ hours after the injection, the excised inoculation site when injected into the monkey caused typical experimental poliomyelitis.

It does not appear probable, therefore, that the rat acts in nature as the reservoir of the virus of poliomyelitis.

[Reprinted from The Journal of Experimental Medicine, October 1, 1918, Vol. xxviii, No. 4, pp. 449–474.]

A REPORT ON ANTIMENINGITIS VACCINATION AND OBSERVATIONS ON AGGLUTININS IN THE BLOOD OF CHRONIC MENINGOCOCCUS CARRIERS.

By FREDERICK L. GATES, M. D.

First Lieutenant, Medical Corps, U. S. Army.

(*From the Base Hospital, Fort Riley, Kansas, and The Rockefeller Institute for Medical Research, New York.*)

Plates 47 and 48.

(Received for publication, July 20, 1918.)

Following an outbreak of epidemic meningitis at Camp Funston, Kansas, in October and November, 1917, a series of antimeningitis vaccinations was undertaken on volunteer subjects from the camp. Major E. H. Schorer, Chief of the Laboratory Section at the adjacent Base Hospital at Fort Riley, offered every facility at his command and cooperated in the laboratory work connected with the vaccinations. In the camp, under the direction of the Division Surgeon, Lieutenant Colonel J. L. Shepard, a preliminary series of vaccinations on a relatively small number of volunteers served to determine the appropriate doses and the resultant local and general reactions. Following this series, the vaccine was offered by the Division Surgeon to the camp at large, and given by the regimental surgeons to all who wished to take it.

Preliminary Series.

The preliminary series of vaccinations was carried out in the 342nd Field Artillery Regiment through the courtesy of Colonel Nugent and Major Czar C. Johnson, surgeon of the regiment. This organization volunteered *en masse* in response to the call issued by the Division Surgeon and offered a most promising opportunity for an extended series of observations. Moreover, only one case of meningitis had developed in the 342nd Field Artillery and the regiment had recently been covered in the search for meningococcus carriers. Dur-

ing the first experience the vaccination of known carriers was avoided and this regiment appeared to be free from them.

Choice and Preparation of Vaccine.—That at least two serologically distinct disease-producing types of meningococci exist has been known since Dopter[1] in 1909 described the "parameningococcus" obtained first from the nasopharynx and then from the blood and spinal fluid of active cases of epidemic meningitis. Following Dopter's discovery Wollstein[2] confirmed the serological distinction between normal or regular meningococci and parameningococci by a comparative study of agglutination, opsonization, and complement fixation of the two types, concluding that:

"Because of the variations and irregularities of serum reactions existing among otherwise normal strains of meningococci it does not seem either possible or desirable to separate the parameningococci into a strictly definite class. It appears desirable to consider them as constituting a special strain among meningococci not, however, wholly consistent in itself."

In a more recent study based on absorption of agglutinin and agglutination tests Gordon[3] has distinguished four groups of meningococci which he calls Types I, II, III, and IV. Groups I and II are distinct and correspond to the known types. They are responsible for 75 to 85 per cent of the cases of epidemic meningitis. Other meningococci he separates into Types III and IV. Tulloch[4] finds, however, that definite group relationships exist between Types I and III, and II and IV, so that a I–III group type and a II–IV group type can be distinguished. These and certain other strains can be classified only by the absorption of agglutinin test, since they agglutinate in two or more of the type sera. Type II also "appears to include a complex subgroup and shows considerable variation among the cocci comprised therein." Nicolle, Debains, and Jouan,[5] employing a rapid method of agglutination or flocculation (without incubation) in low dilu-

[1] Dopter, C., Étude de quelques germes isolés du rhino-pharynx, voisins du méningocoque (paraméningocoques), *Compt. rend. Soc. biol.*, 1909, lxvii, 74.

[2] Wollstein, M., Parameningococcus and its antiserum, *J. Exp. Med.*, 1914, xx, 201.

[3] Gordon, M. H., The definition of the meningococcus, *National Health Insurance, Med. Research Committee, Special Rep. Series, No. 3*, London, 1917, 10.

[4] Tulloch, W. J., A study of the mechanism of the agglutination and absorption of agglutinin reaction, together with an examination of the efficacy of these tests for identifying specimens of the meningococcus isolated from 354 cases of cerebrospinal fever, *J. Roy. Army Med. Corps*, 1918, xxx, 115.

[5] Nicolle, M., Debains, E., and Jouan, C., Sur lesméningocoques et les sérums antiméningococciques, *Bull. et mém. Soc. méd. hôp. Paris*, 1917, xli, 878; *Ann. Inst. Pasteur*, 1918, xxxii, 150.

tions of immune horse serum, have also reported four types of the meningococcus which they designate A (Gordon I and III, regular meningococcus), B (Gordon II and IV, parameningococcus), C, and D. Types A and B are common, Types C and D relatively rare. They find that certain meningococci are related to two or more of their specific types and must therefore be classed as "indeterminates."

In a recent article Mathers and Herrold[6] readily distinguish the two main types by agglutination, and group around them most of their atypical strains. The strains which fail to agglutinate in type sera have nevertheless similar antigenic properties, since "monovalent serum prepared from these strains agglutinates in a specific way meningococci belonging to one or the other of the large biologic groups."

It appears, therefore, that while two distinct types or groups of meningococci may be clearly differentiated, most of the other organisms that do not fall strictly within these two groups are either intermediates or irregular variants of one or the other. Meningococci of other types in as far as they can be distinguished are only occasionally responsible for a case of epidemic meningitis.

In order to cover the two pathogenic types of meningococci and variants of them, it has become the practice in this country, following the methods of Flexner and Jobling[7] and Amoss and Wollstein,[8] to prepare highly polyvalent therapeutic sera by the repeated injection of a number of representative strains with the object of producing a serum which would protect against any pathogenic strain that might be encountered. For protective inoculation high polyvalency would seem to be equally desirable, but the choice of strains for human vaccination is limited by the fact that only a few injections are practicable, whereas the production of therapeutic serum requires a long series of inoculations in which immunity is gradually built up by the repeated injection of small doses of each antigen. The inclusion of any considerable number of strains in a vaccine for human use might defeat the purpose of the vaccination by the introduction of toxic amounts of bacterial protein without a sufficient quantity of any one specific

[6] Mathers, G., and Herrold, R. D., Observations on meningococcus carriers and on the bacteriology of epidemic meningitis, *J. Infect. Dis.*, 1918, xxii, 523.

[7] Flexner, S., and Jobling, J. W., Serum treatment of epidemic cerebro-spinal meningitis, *J. Exp. Med.*, 1908, x, 141.

[8] Amoss, H. L., and Wollstein, M., A method for the rapid preparation of anti-meningitis serum, *J. Exp. Med.*, 1916, xxiii, 403.

antigen to give rise to protection against its given strain. But since 70 to 85 per cent of the cases of epidemic meningitis are caused by meningococci of the two main types, the limitation of the strains in a vaccine to representatives of these types would seem to be the rational procedure, holding out hope of protection against a large proportion of disease-producing strains.

Therefore, for the vaccine used at Camp Funston only three organisms were chosen from the stock of The Rockefeller Institute, but the vaccine may be regarded as having represented the two main types of the meningococcus. It was also anticipated that the epidemic at Camp Funston might furnish some strain or strains of meningococci not closely related to the main types and which ought therefore to be included in the vaccine, but a type study at Fort Riley showed that the sixteen strains recovered from active cases in December and January would be covered by a vaccine containing normal and para strains.

Method of Preparing Vaccine.—The vaccine used was made in the laboratory of The Rockefeller Institute. 16 hour growths on 1 per cent glucose agar in Blake bottles were washed off with isotonic salt solution, like strains pooled, and the concentrated suspensions immediately heated to 65°C. for 30 minutes to kill the cocci and inactivate the autolytic ferment. Experiments have shown that this temperature does not impair the antigenic properties of the organism and the intact cocci are less toxic than their autolyzed products.

Following the usual tests for purity and sterility the suspensions were standardized, diluted in 0.85 per cent salt solution, mixed in equal proportions of the three strains in concentrations of 1,000 million, 2,000 million, 4,000 million, and 8,000 million cocci per cc. and preserved with 0.35 per cent tricresol.

All the injections in the preliminary series were made strictly subcutaneously with a fine needle (No. 25) at the insertion of the deltoid muscle, usually in the left arm unless a recent vaccina "take" had occurred. Then the right arm was used. The tincture of iodine used as antiseptic was then sponged off with alcohol.

For the determination of dosage and the study of reactions and antibody formation six groups of about 50 men each were chosen from the various companies in the regiment. Successive groups received

increasing doses of vaccine in a series of three injections at 4 to 10 day intervals according to the schedule in Table I.

About 25 men of Groups I, II, III, V, and VI gave preliminary (control) blood samples for the study of immunity reactions on the day of the first injection and a later specimen was obtained from as many vaccinated men as were available on the 8th to 10th day after the third injection.

At Major Johnson's direction First Lieutenant Serge Androp, Medical Corps, obtained reports from the men at inspection the morning following the vaccinations in regard to the resulting local and general reactions.

TABLE I.

Vaccination Schedule, Preliminary Series.

Group No.	1st injection.	Interval.	2nd injection.	Interval.	3rd injection.	Interval. Blood samples taken.
	millions	*days*	*millions*	*days*	*millions*	*days*
I	500	4	1,000	4	2,000	8
II	750	9	1,500	10	3,000	9
III	1,000	9	2,000	10	4,000	9
IV	1,500	8	3,000	8	8,000	10
V	2,000	7	4,000	7	8,000	10
VI	2,000	6	5–6,000	6	10,000	9
VII	2,000	7	4,000	8	8,000	17

Dosage and Reaction.—The determination of the dosage of vaccine for subsequent groups followed from the reports of the reactions produced by the given doses. It was considered important to increase the doses gradually in order to locate closely the zone of mild reactions and to avoid unexpectedly severe results. Accordingly, the vaccinations were begun with the injection of 500 million cocci, and this initial dose was increased in successive groups by 250 or 500 million until it had reached 2,000 million. For the second and third doses in each group, the first dose was usually multiplied by two and by four. Thus it usually happened that a given dose had already been used as a second or third in one group before it was tried as the first dose in a later series.

Only two reactions of any consequence were reported from doses of less than 2,000 million cocci. An officer in Group II who explained

that he had long been hypersensitive to foreign protein and had reacted severely to the typhoid and paratyphoid vaccinations developed a severe local and general reaction, with headache and malaise, after the first injection of 750 million cocci. A second officer in Group II was similarly affected after a second injection of 1,500 million cocci and was confined to bed part of the following day. The two reactions after these small doses were merely transient, but they demonstrate a factor of individual susceptibility which was found to be of considerable importance in the determination of proper doses of vaccine.

When an initial dose of 2,000 million was reached (Group V) general reactions began to appear with greater frequency. None of the men in this group felt ill enough to report at sick call the following morning, but nine men stated upon question that they had felt feverish the preceding night. Four of them had had headache also and one reported a chill. Arms were moderately sore at the site of injection, but not sore enough to interfere with routine duties.

From this time on, a small number of the men in each group reported some local or general discomfort following the vaccination. The symptom most frequently mentioned was a "feverish sensation" often accompanied by headache, which was sometimes severe enough to cause loss of sleep. Morning temperatures, when taken, were reported normal. In a few instances there was transient nausea, malaise, or aching joint pains, and three reactions were initiated with a chill. Eight men had general reactions after the first and second doses, or after the second and third, and three complained of discomfort after all three injections.

On the whole the reactions produced by the vaccine in the preliminary series were mild as compared with those that occasionally follow injections of typhoid or paratyphoid prophylactic and there was little complaint among the men. The occurrence of an occasional reaction of greater severity even with the smaller doses, and increasing local tenderness after the injection of the larger doses of vaccine led to the choice of relatively lower doses for the general series throughout the camp rather than the attempt to push the dosage up to the limit of endurance. Later experience fully justified this decision. Doses of 2,000, 4,000, and 8,000 million had already been decided upon when Group VI was given a second injection of 5,000 or 6,000

million and a third injection of 10,000 to determine the relative security of the chosen doses. The injection of 10,000 million cocci caused general reactions in eleven men, but none of them was confined to bed or relieved from duty following the injection.

Finally, a seventh group of 99 men received the chosen doses of vaccine at weekly intervals about a week before the corresponding injections were given in the camp at large and served as a final check on the dosage. On giving the third injections to Group VII it was noted that many of the men still had a small painless area of subcutaneous induration at the site of the former injections. This persistent induration has been personally experienced after injection of meningococcus vaccine. It disappears gradually, leaving no trace. A more general discussion of reactions is reserved for the report on the larger vaccination series.

Immunity Reactions.—Agglutination is the reaction of choice for the study of antibody production with the meningococcus. It is the most delicate as well as the most specific, and is most easily made on a large number of sera and in multiple dilutions. In studying agglutination, however, several factors have to be taken into account aside from the obvious requirement of a carefully standardized technique. One of the most important is the relative agglutinability of various strains of meningococci and their response to one or both of the type (normal and para) immune bodies. Meningococci vary also in antigenic power and not always in consonance with their agglutinability, so that it is sometimes profitable to use certain type strains as antigens and other strains to test antibody production.

Most of the control sera taken in advance of vaccination from men of Groups I, II, III, V, and VI were used up in agglutination tests against the vaccine strains in a dilution of 1:10. Except in one instance in which the para strain was partially agglutinated, the results of these tests were uniformly negative. When the first of the serum specimens, taken on the 8th and 10th days after vaccination and tested in 1:10 dilution against the vaccine strains, also gave negative results, it was decided to collect all the available sera for later study in lower dilutions and against more easily agglutinable strains. This study was subsequently made at The Rockefeller Institute. A certain number of sera from each group was examined by macroscopic

agglutination with a modification of the Wright method in dilutions of 1:2 and higher against the following strains from the stock of The Rockefeller Institute:

No. 8. A "regular" which is also agglutinated in low dilutions of para serum.
No. 10. An intermediate which agglutinates in both "regular" and para serum.
No. 60. One of Dopter's paras which is relatively easily agglutinable and was represented in the vaccine.

On account of the limited amounts of serum available and the low dilutions required, the following method of agglutination was employed:

Capillary glass tubing of an internal diameter of 2 mm. is drawn out into capsules 8 to 10 cm. long (Fig. 1). For use the ends are snapped off and a nipple is slipped on one end and folded double, giving accurate control of the aspirated fluids. A file mark measures equal volumes of serum and salt solution for successive dilutions, which are made in the capsule and deposited in a row on a plate of solid paraffin in a Petri dish. The paraffin plate is conveniently cleaned by melting off a thin layer in hot water. Equal amounts of a serum dilution and a meningococcus suspension are measured in the capsule, mixed on the paraffin plate, and drawn up to form a column about 1 to 1.5 cm. long. Four or five such specimens, separated by air bubbles, are sealed in a capsule for incubation. Only one dilution of a serum should be used in a single capsule, but several meningococcus suspensions (4 billion per cc.) may be tested against it, as the admixture of specimens in the capsule is inappreciable. The capsules are woven through holes in a card which designates their contents, and incubated in a horizontal position for 24 hours at 55°C. Complete agglutination is indicated by a widespread flocculated sediment of organisms (Fig. 2). The flocculi are distributed through the clear fluid on rolling the tube briskly between the palms (Fig. 3). Absence of agglutination corresponds to a smooth line of sediment, which goes into even suspension on whirling. Partial agglutination is easily recognized in a combination of the two types of sediment. Results are read with the unaided eye or under a small hand lens.

As stated above, most of the control sera had been exhausted before this more comprehensive series of tests was undertaken. There remained fourteen normal control sera from Group VI, and it happened that the practically negative results obtained with the sera of Groups I, II, and III admit these specimens as controls, since the small doses of vaccine used in these groups did not give rise to demonstrable antibody formation. To these may be added twelve sera from supposed non-contacts obtained in New York City.

TABLE II.

Agglutination Tests with Sera of Vaccinated Men.
Dilution 1:2.

Controls, not vaccinated.	Strains.			Group I.	Strains.			Group II.	Strains.		
	8	10	60		8	10	60		8	10	60
N 1	−	−	−	5	++	++	++	71	−	−	−
N 2	−	−	−	8	−	−	−	73	−	−	−
N 3	−	−	−	11	−	−	−	77	++	++	−
N 4	−	−	+	13	−	−	−	79	−	−	−
N 5	−	−	−	14	−	+	−	81	−	−	−
N 6	−	−	−	15	−	+	−	88	−	−	−
N 7	−	−	−	20	−	−	−	94	−	−	−
N 8	−	−	−	21	+	−	−	100	−	−	−
N 9	−	−	−	24	−	++	−	112	−	−	−
N 10	−	−	−	25	−	−	−	113	−	−	−
N 11	+	−	−					114	−	+	−
N 12	−	−	−					115	−	−	−
Group III.				**Group IV.**				**Group V.**			
122	−	−	−	173	+	+	+	227	−	++	+
125	−	−	−	179	−	+	−	233	++	++	+
126	−	−	−	180	−	+	−	234	++	++	−
127	−	−	−	183	−	+	−	237	+	++	+
134	−	−	−	185	−	++	−	239	+	+	+
135	−	−	−	186	−	−	−	240	++	++	−
140	−	−	−	188	−	−	−	246	−	+	−
142	−	−	−	192	−	+	−	250	−	++	+
147	−	−	−	195	−	+	−	251	−	++	++
152	−	−	−	197	−	−	−	252	++	++	++
155	−	−	−	202	−	++	−	253	−	++	+
160	−	−	−	206	−	+	−	254	−	++	+
				210	−	+	−	255	−	++	+
				212	−	++	−	256	++	++	+
				215	−	+	−	257	−	++	++
								259	−	+	−
								261	++	++	+
								262	+	++	++
								263	++	++	++
								264	++	++	++
								267	−	++	++
								268	−	++	++

++indicates complete agglutination, + partial agglutination, − no agglutination.

TABLE II—*Concluded.*

Group VI.	Before vaccination (controls). Strains.			After vaccination. Strains.			Group VII.	Strains.		
	8	10	60	8	10	60		8	10	60
269				−	−	−	322	−	++	++
270				−	+	+	323	−	++	++
271	−	+	+	−	+	−	324	−	+	++
273	+	+	−	−	++	++	326	−	++	++
274				+	++	−	331	−	+	+
278	−	−	−	+	++	−	334	−	++	++
284	−	−	−	−	+	−	335	−	++	++
286	−	−	−	+	++	+	354	−	++	++
287	−	−	−	−	++	−	380	−	++	++
288	−	−	−	−	+	−	395	−	+	+
289	−	−	−	−	+	−	397	−	++	++
290	+	+	−	+	++	+	399	−	++	+
291	−	−	−	+	+	−	406	−	++	+
292	−	−	−	−	++	−	409	−	++	++
294	−	−	−	−	+	−	415	+	++	++
296	−	−	−	+	++	++				
299				−	+	+				
310				−	+	+				
317				−	+	−				

Table II shows the absence of agglutination in Groups I, II, and III with few exceptions. In three cases only was more than a partial agglutination of one strain in the 1:2 dilution observed. The titers in these cases ran as follows:

Group.	Serum.	Strain.		
		8	10	60
I	5	1:32	1:8	1:8
	24	0	1:32	0
II	77	1:8	1:32	0

It may be assumed, therefore, that the number of unvaccinated men whose sera would agglutinate any of these chosen strains of meningococci is small, and on the basis of this assumption the observation that twelve of fifteen sera from Group IV agglutinate Strain

10 partially or completely is unmistakable evidence of antibody formation through the agency of the vaccine. Group V, in which the dosage reached that chosen for the camp at large, confirms this evidence of reaction, for all the twenty-two sera studied contained antibodies for the meningococcus. In some instances the more easily agglutinable strains were agglutinated in a dilution of 1:32 or 1:64 (Table III).

TABLE III.

Agglutinin Titers of Sera from Group V.

Serum.	Strains.		
	8	10	0
227	0	1:32	1:32
233	1:2	1:32	1:8
234	1:2	1:4	0
237	1:2	1:16	1:4
239	1:2	1:16	1:2
240	1:2	1:64	0
246	0	1:2	0
250	0	1:128	1:8
251	0	1:32	1:32
252	1:32	1:64	1:8
253	0	1:32	1:8
254	0	1:64	1:8
255	0	1:32	1:2
256	1:2	1:64	1:2
257	0	1:64	1:16
259	0	1:2	0
261	1:2	1:32	1:2
262	1:2	1:32	1:32
263	1:4	1:32	1:32
264	1:2	1:64	1:64
267	0	1:8	1:8
268	0	1:64	1:8

The final evidence of antibody production is furnished by a direct comparison of sera from Group VI taken before the first vaccination with sera taken 9 days after the third injection of vaccine, in which the appearance or increase in agglutinins is observable in all but two instances. The presence of agglutinins in the sera from Group VII merely confirms the previous findings. *It may, therefore, be stated*

that the injection of well tolerated doses of meningococcus vaccine is followed by specific antibody formation in the human body.

The preliminary series of vaccinations, therefore, served to establish the method of injection, the proper dosage for extended vaccination, the reactions which might be expected to follow the chosen doses, and the production of immune bodies in the serum of vaccinated men. On the basis of these findings the vaccine was offered to the camp at large.

General Series.

The vaccine used in the general series of inoculations in the camp was made by Lieutenant Peter K. Olitsky at The Rockefeller Institute by the methods already described. It was shipped to Fort Riley in bulk and was diluted in isotonic salt solution to standard concentration, preserved with 0.3 per cent cresol and distributed in 50 cc. bottles to the regimental surgeons under Major Schorer's direction. The use of two suspensions, one of 4,000 million cocci per cc. (Vaccine A and B), and the other of 8,000 million (Vaccine C), adjusted the injection volumes to 0.5 and 1 cc. of Vaccine A and B and then 1 cc. of Vaccine C, volumes similar to those for the typhoid prophylactic, with doses of 2,000 million, 4,000 million, and 8,000 million cocci to be given at weekly intervals.

At the direction of the Division Surgeon, the Division Training Officer, Captain Albert Bower, to whom especial thanks is due for his mediation between the regimental surgeons and the laboratory, called a meeting of the surgeons at which the method of giving the vaccine and the results to be expected and observed were fully described and discussed. The surgeons were thus informed of the procedure and object of the vaccination and their cooperation was enlisted, without which little could have been accomplished. The response of the men when the vaccine was offered to them was due in large measure to the interest and example of the regimental surgeons.

According to the statistics of the division headquarters, the total strength of the 89th Division at this time was approximately 25,000 officers and men. Of these, 4,792 (19 per cent) took the first injection, 4,257 (17 per cent) the second also, and 3,702 (15 per cent) completed the series.

Part of the men received the full dosage as planned. About half of those vaccinated, whose third injection was due after February 4, 1918, were given a final injection of 4,000 million, on account of the occurrence of several fairly severe reactions from the larger dose among medical officers at Fort Riley. In some regiments the vaccinations had been completed before February 5.

Reactions.—After the first injections had been given, the opinion was almost universal in the camp that the vaccine caused less general and local reaction than the typhoid prophylactic. In very few regiments was a man excused from duty the following day on account of the reaction from the vaccination. The general feeling was that the second dose caused less reaction than the first, but there were a few men in almost every organization who had reactions of moderate severity, sometimes being confined to bed for the day with headache, joint pains, and nausea. Several cases of looseness of the bowels or transient diarrhea were noted. This symptom had not been encountered before. Careful inquiry in individual cases often elicited the information that men who complained of the effects of vaccination were suffering from mild coryza, bronchitis, etc., at the time of injection.

Among the units who took the third injections before the dosage was reduced, and so received a third dose of 8,000 million meningococci, there were several instances of fairly severe reactions, general and local, which necessitated relief from duty the following day. The reactions were not more severe than those that occasionally follow paratyphoid prophylactic and no untoward results were reported. The large majority of the men seem to have suffered no appreciable reaction whatever. The smaller doses of 4,000 million cocci caused even fewer reactions.

As in the preliminary series, the factor of individual susceptibility was prominent, a few officers and men suffering severely from doses which caused no general discomfort in the great majority of the men. In general, the more severe reactions occurred among the commissioned officers, and especially among the medical officers at the Base Hospital and in the Medical Officers' Training Camp at Fort Riley, due in part perhaps to more confining occupations, higher nervous tension, and more introspection than was common among the enlisted

men. In one regiment, through a misunderstanding, four men were given an initial dose of 8,000 million cocci, which was well tolerated. The surgeon reported:

"One had chill for 30 minutes, headache for 1 hour, slight local reaction.
One had slight headache, slight local reaction.
One had severe local reaction and headache for 24 hours.
One had slight local reaction, headache for 12 hours."

A survey of the reports of the regimental surgeons and of the observations in the preliminary series shows that headache was the most frequent symptom following injection, and accompanied most of the other symptoms encountered. Sometimes the reaction was initiated by a chill or chilly sensation, and a number of men complained of fever or feverish sensations during the following night. Next in frequency came nausea (occasionally vomiting), dizziness, and general "aches and pains" in the joints and muscles, which in a few instances were especially localized in the neck or lumbar region, causing stiff neck or stiff back. A few injections were followed by diarrhea. The reactions, therefore, occasionally simulated the onset of epidemic meningitis and several vaccinated men were sent as suspects to the Base Hospital for diagnosis.

Such transient reactions are illustrated by the following brief protocols:

Individual 1.—C. D., Private, Battery D, 342nd Field Artillery.

Jan. 15, 1918. 1st injection 2,000 million. "Sore arm."

Jan. 22. 2nd injection 4,000 million. "Sore arm, headache."

Jan. 29. 3rd injection 8,000 million. "Began to feel badly about 15 minutes later and had a chill. 3 hours later was sent to the Base Hospital complaining of headache, lumbar pain, stiff neck, and fever, 103°F. No nausea. Went to sleep about 2 hours later and slept well. Had entirely recovered the next morning."

Individual 2.—L. N., Private, Field Hospital Company 354, 314th Sanitary Train.

Jan. 21, 1918. 1st injection 2,000 million. "Slight local soreness."

Jan. 28. 2nd injection 4,000 million. "Slight local soreness."

Feb. 4. 3rd injection 8,000 million. "About an hour and a half after injection, was taken with a severe chill and a subnormal temperature. About 20 minutes later his temperature rose to 103.6°F. and then fell within the next 2 hours to 101°F. Temperature normal the following morning. He complained of no other symptoms."

The most severe illness immediately following vaccination is described by the officer himself as follows:

Individual 3.—J. M. K., First Lieutenant, 314th Sanitary Train.

Jan. 21, 1918. 1st injection 2,000 million. 4.30 p.m. Soreness at site of injection in evening. Slept well.

Jan. 22. Awakened feeling drowsy and listless. Local soreness worse. Pains in back of neck, calves of legs, thighs, lumbar region, and both arms. No appetite. Symptoms grew worse after holding sick call at the Infirmary. Perspired on slight exertion. Felt chilly and warm by turn. Went to quarters and lay in bunk. Soon felt feverish. Developed diarrhea about 11 a.m. Eight movements in 5 hours. Cup of hot chocolate at 1 p.m. Felt nauseated. Vomited six times in next 3 hours. Took sodium bicarbonate, 2 gm. in glass of warm water, promptly vomited. Retained 15 minutes later, and then began to feel better. Felt well next morning but had no appetite. Bowels still loose. Returned to duty. Appetite returned the evening of Jan. 24. Local soreness persisted 3 days.

		Temperature. °F.	Pulse.
Jan. 22,	9.45 a. m.	98.4	86
	1.1 p. m.	99.8	82
	3.45 p. m.	101.0	92
	7.30 p. m.	101.8	108
	10.00 p. m.	100.0	98
Jan. 23,	7.25 a. m.	97.8	98

Jan. 28. 2nd injection 4,000 million. Local reaction severe. Duration 3 days. General reaction, malaise of 24 hours duration.

Feb. 4. 3rd injection 8,000 million. Local reaction severe. Duration 4 days. General reaction, nausea, looseness of bowels (not diarrhea), chilliness, but no fever. Muscle soreness severe and general. Appetite absent for 2 days.

The occurrence of such reactions as those described above was rare but they illustrate the importance of the factor of individual susceptibility. The great majority of the men vaccinated were reported to have had no general symptoms, and very few men were confined to quarters after any dose of the vaccine.

Occurrence of Meningitis after Vaccination.—The records of cases of epidemic meningitis in Camp Funston have been followed up to June 4, 1918, thus covering the period that the 89th Division remained in camp. In the interval between January 21 when the vaccination was started in the camp and June 4, 46 cases of meningitis are reported to have entered the Base Hospital at Fort Riley. Of these patients, three had received one, two, or three injections of antimeningitis vaccine. The following data have been collected in regard to these cases.

Case 1.—L. T., Second Lieutenant, 314th Engineers.

Jan. 21, 1918. 1st injection 2,000 million. Next day neck was rather stiff. slight headache. Well for next 2 days, but working very hard on double duty.

Jan. 25. Fainted at Officers' Call. Felt "under the weather" with indefinite symptoms the next 2 days but remained on duty.

Jan. 28. 2nd injection 4,000 million. This made him feel so much better that he remarked to his friends that he "was getting to be a dope fiend in the stuff and couldn't get along without it."

Jan. 29. Felt worse again, headache, "no ambition." Following days he remained on duty, but tired easily and often felt chilly and feverish, had stiff neck and joint pains.

Feb. 2. Remained in bed. Throbbing headache, became nauseated and vomited twice. Sent to Base Hospital. Lumbar puncture on arrival showed a turbid fluid with many leukocytes and moderate numbers of intracellular Gram-negative diplococci.

Lieutenant T. had a very mild case of meningitis which yielded promptly to serum treatment.

Case 2.—E. H., Corporal, Company C, 354th Infantry.

Jan. 21, 1918. 1st injection 2,000 million. "Never felt anything. Arm not very sore."

Jan. 26. Went on 5 days leave, returning Jan. 31, and so missed second injection.

Feb. 1. "Felt sore all over." Had chills. Frontal headache.

Feb. 2. Headache continued, stiff joints and aching pains. Sent to Base Hospital. Diagnosis, epidemic meningitis.

Corporal H. had a severe case of meningitis but recovered.

Case 3.—J. C. N., Private, Company B, 340th Machine Gun Battalion.

Jan. 24, 1918. 1st injection 2,000 million.

Jan. 31. 2nd injection 4,000 million.

Feb. 8. 3rd injection 4,000 million. Reactions not reported.

Mar. 31. Acute otitis media and right frontal sinusitis.

Apr. 16. Epidemic cerebrospinal meningitis.

Apr. 24. Cerebrospinal fluid found positive for meningococcus for the last time.

Apr. 27. Ulcer of right cornea followed by slight opacity with slight impairment of vision.

May 5. Right facial paralysis which has since improved considerably.

Private N. will be ready for assignment to domestic military service about July 1, 1918.

The examples of Lieutenant T and Corporal H are instructive, since they may well be, and probably are, instances of meningococcus inoculation in individuals in the incubation period of a meningococcus meningitis. Assuming this to be the fact, the indication is that no

harm was done by the procedure. But whether this is the precise fact or not, the cases have no real bearing on the value of antimeningitis vaccination, because the interval between the first inoculation and

TABLE IV.

Agglutination of Meningococci in Sera of Vaccinated Men.
Dilution 1: 2.

314th Engineers.	After 3rd injection.	Strains.			355 Infantry.	After 3rd injection.	Strains.			314th Sanitary Train.	After 3rd injection.	Strains.		
		8	10	60			8	10	60			8	10	60
	days					*days*					*days*			
501	17	−	+	−	535	15	−	−	−	585	17	−	++	+
502	17	−	+	−	536	15	−	++	+	586	17	−	+	+
503	17	−	+	+	537	15	+	++	++	587	17	−	−	−
504	17	−	−	−	538	15	−	++	+	590	17	+	−	−
506*	10	−	++	++	539	15	−	+	−	591	17	−	−	−
509*	10	−	−	−	541	15	−	++	+	593	17	−	+	+
510*	10	−	−	−	544	15	−	+	−	595	17	−	++	++
511*	10	−	++	++	545	15	−	++	+	598	17	+	++	++
512*	10	−	++	+	547	15	+	++	++	600	17	−	+	+
513*	10	−	++	−	549	15	+	+	+	606	17	++	++	++
514*	10	−	+	+	553	15	++	++	++	609	17	−	++	++
515*	10	−	+	−	558	15	+	++	+	610	17	++	++	++
516*	10	−	+	++	560	15	−	−	−	611	17	+	++	+
517*	10	−	−	−	562	15	+	+	−	612	17	−	++	++
518*	10	−	−	−	565	15	−	+	−	613	17	++	++	++
520*	10	+	++	++	566	15	−	−	−	614	17	−	++	++
528	18	−	−	−	568	15	−	−	−	615	17	−	−	−
531*	10	−	+	+	569	15	−	++	+	618	17	+	++	++
532	18	−	+	−	570	15	−	−	−	619	17	−	++	++
533	18	−	−	−	571	15	−	+	−	622	17	−	+	+
534	18	−	−	−	574	15	−	++	+					
					576	15	−	++	++					
					578	15	+	++	+					
					581	15	−	++	+					
					583	15	−	+	+					

* 3rd dose 4,000 million cocci.

the diagnosis of the meningitis was too brief to permit immunization to be effected.

It is clear that Private N developed meningitis at a period at which protection following vaccination should have been present. In his case the vaccination must be considered as having failed to afford

protection. A study of the type of the infecting meningococcus would conceivably have thrown light on the incident, or the incident may merely indicate that occasional individuals fail to be adequately immunized, just as in the analogous procedure of antityphoid inoculation.

Immunity Reactions, General Series.—In addition to the blood samples taken for study in the preliminary series of vaccinations, similar samples were obtained from a group of men in each of three regiments who had completed the vaccination series. The serum specimens were taken at an interval of from 10 to 18 days after the third injections had been given, and were examined for agglutinins by the method already described. The results of these tests are shown in Table IV. The sera of 50 men showed some content of agglutinins; the sera of 16 others failed to do so.

Examination for Agglutinins of the Blood Serum of Chronic Carriers.

While every case of epidemic meningitis is presumed to develop from the carrier state, many observers have noted the rarity of the disease among chronic carriers. The presence of the meningococcus in the nasopharynx is but one of the factors in the accident which results in the bacterial invasion of the body, and the relatively low infectivity of the meningococcus is to be credited to causes of resistance on the part of the carrier host among which may well be the appearance of immune bodies in the blood as the result of the multiplication of meningococci in the nasopharynx.

If the presence of agglutinins for the meningococcus could be demonstrated in the blood of chronic carriers, light would thereby be thrown on the mechanism of this resistance. With the object of detecting the possible existence of meningococcus agglutinins in the blood serum of carriers, a number of such sera have been studied in low dilutions by the capsule method described in an earlier section of this report.

Through the courtesy of Passed Assistant Surgeon J. L. Waterman, United States Naval Hospital, Brooklyn Navy Yard, and Dr. F. S. Westmoreland, Riverside Hospital, New York, opportunity was given to obtain blood samples from a number of chronic carriers of the minengococcus, and the corresponding carrier strains were kindly furnished

by Dr. A. W. Williams of the Department of Health, of the City of New York, or obtained directly from the men themselves. The men studied were known to have harbored meningococci for from 4 to 16 weeks.

20 carrier strains of the meningococcus have been subjected to the agglutination tests in low dilutions with the sera of the hosts from whom they were obtained and the carrier sera were tested against the stock strains of spinal origin that had been studied in the sera of vaccinated men from Camp Funston. 18 of the carrier strains were also

TABLE V.

Control Agglutination Tests with Normal Sera against Carrier Strains. Dilution 1: 2.

Normal control serum.	Carrier Strains.																	
	124	125	127	128	129	133	134	136	139	141	147	150	151	153	156	160	161	162
1	−	−	−	−	−	−	−	−	−	++	+	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	+	−	++	++	−	−	−	−	−	−	−
4	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
5	+	−	+	−	−	−	−	−	−	++	+	−	−	−	−	−	−	−
6	−	−	−	−	+	−	−	−	−	+	+	−	−	−	+	−	+	+
7	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−	*	−	−	+	−	−
9	++	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
10	+	+	+	+	+	+	−	−	−	++	++	−	−	−	++	−	+	+
11	+	−	−	−	−	−	+	−	−	+	+	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

*No test.

tested with 12 normal sera from supposed non-contacts as controls. The results of these tests are recorded in Tables V to IX. The trials with non-contact sera (Table V) show that with two exceptions the carrier strains are not usually agglutinated in normal sera in a 1: 2 dilution. One of the exceptional strains was partially or completely agglutinated by 9 and the other by 8 of the 12 sera. The other cultures were only occasionally agglutinated in this low dilution of normal serum. One supposedly normal serum agglutinated 11 of the 18 carrier strains. In the presence of sera from their hosts, however,

all but 4 of these carrier strains were agglutinated (Table VI) and the sera in most instances showed agglutinins for stock spinal strains of the meningococcus. Subsequently the sera were reexamined in multiple dilutions, and at this time 2 of those previously found negative agglutinated their strains up to 1:4 and 1:8 (Table VII). The titers of the other sera ran from 1:4 to 1:64.

TABLE VI.

Agglutination of Homologous Nasal Strains and Stock Spinal Strains in Sera of Carriers.

Serum of.	Homologous nasal strains.	Spinal Strains.		
		8	10	60
Carrier 2	++	++	++	++
" 3	−	−	−	−
" 6	+	+	++	++
" 9	++	++	++	+
" 11	+	++	++	−
" 13	++	++	++	++
" 16	++	++	++	++
" 18	+	++	++	+
" 25	+	++	++	+
" 27	++	++	++	++
" 28	++	++	++	++
" 29	++	++	++	++
" 30	+	+	++	+
" 36	+	−	−	−
" 39	++	++	+	−
" 40	+	+	+	−
" 41	−	+	−	−
" 43	−	+	−	−
" 48	++	++	++	−
" 50	−	−	−	−

Appearance of Agglutinins in Sera after Storage.—On numerous occasions we have observed an increase in agglutinating power in sera which have stood at ice box temperature for some time. The fact was reported in a recent paper[9] as having occurred with typhoid agglutinins in guinea pig sera, and Tulloch[4] records an instance in

[9] Gates, F. L., Antibody production after partial adrenalectomy in guinea pigs, *J. Exp. Med.*, 1918, xxvii, 725.

TABLE VII.

Agglutination of Carrier Strains in Homologous Sera.

Serum of.	Homologous Strain.	Agglutination titers.					
		1:2	1:4	1:8	1:16	1:32	1:64
Carrier 2	124	++	+	+	−	−	−
" 3	125	+	+	+	−	−	−
" 6	127	+	++	+	++	−	−
" 9	128	++	++	++	++	++	+
" 11	129	+	++	++	++	++	++
" 13	133	++	+	−	−	−	−
" 16	134	++	++	++	+	−	−
" 18	136	++	++	+	+	−	−
" 27	139	++	++	+	+	−	−
" 28	141	++	++	+	+	−	−
" 36	147	++	+	+	+	++	++
" 39	150	++	++	++	++	+	+
" 40	151	−	+	+	+	−	+
" 41	153	++	*	+	−	−	−
" 43	156	−	−	−	−	−	−
" 46	160	++	++	++	++	++	++
" 48	161	++	++	++	−	−	−
" 50	162	++	+	−	−	−	−

* No specimen.

TABLE VIII.

Appearance of Agglutinins in Carrier Sera after Storage.
Dilution 1: 2.

Serum drawn May 24, 1918.	Examination of May 29.				Examination of June 12.				Examination of June 24.			
	Homologous Strain.	Spinal Strains.			Homologous Strain.	Spinal Strains.			Homologous Strain.	Spinal Strains.		
		8	10	60		8	10	60		8	10	60
16	−	−	−	−	+	++	++	+	++	No test.		
36	−	−	−	−	+	−	−	−	++	−	+	+
39	−	−	−	−	++	++	+	−	++	−	++	++
40	−	−	−	−	+	+	+	−	−*	−	−*	−
41	−	−	−	−	−	+	−	−	++	−	++	++
43	−	−	−	−	−	+	−	−	−	−	++	++
46	−	−	−	−	No test.				++	−	−	++
50	−	−	−	−	−	−	−	−	++	−	+	+

* + in higher dilutions.

rabbit serum during immunization to the meningococcus. This he explains as due to the presence of constituents of the serum or of the organisms which do not take part in the mechanism of agglutination, but which may be present "in such quantity or in such a physical state that they protect the united antibody-antigen complex from the flocculating action of the electrolytes."

Table VIII shows the appearance of agglutinins in some of the sera under discussion after they had stood, sealed, at 4°C. for some time. The other sera were not examined so early after bleeding. The

TABLE IX.

Agglutination of Stock Strains in Sera of Carriers.

Serum of.	Agglutination titers of homologous strains in rabbit serum.		Agglutination titers of stock spinal strains in carrier sera.		
	Normal.	Para.	No. 8	No. 10	No. 60
Carrier 27	1:400+	1:400	1:2	1 16	1:16
" 28	1:400+	1:100	0	1:32	1:4
" 36	1:400+	1:50	0	1:64	1:4
" 39	1:800	1:100	0	1:32	1:64
" 40	1:400+	1:50	0	1:64	0
" 41	1:400+	1:100	0	1:32	1:8
" 43	1:50	1:100	0	1:32	1:8
" 46	1:800	1:200	0	0	1:16
" 48	1:50	1:100	0	1:32	1:8
" 50	1:400+	0	0	1:8	1:2

inconstancy of the phenomenon has already been described[9] and is implied by Tulloch in his reference to "this particular serum."

A variation of a different order was encountered when ten of the sera studied on May 29 or June 12 were examined on June 24 in multiple dilutions with the stock spinal strains (Table IX). Six of the ten had agglutinated Strain 8 in the 1: 2 dilution. Now all but one serum failed to do so. Strains 10 and 60, however, were agglutinated in various serum dilutions by practically all the specimens examined, including No. 43, which had failed to agglutinate its homologous strain. Agglutinins for these spinal strains are only rarely found in normal sera (Table II).

DISCUSSION.

Heretofore meningococcus vaccines have not been extensively employed for prophylactic immunization, and only a few references are to be found in the literature that relate vaccination experiences. Davis,[10] in 1907, describing animal experiments and the therapeutic use of an autogenous vaccine reported a personal experience in which he suffered a very severe reaction following the subcutaneous injection of a 24 hour slant culture of a meningococcus heated to 65°C. for 30 minutes. Shortly after inoculation, nausea and vomiting were followed by a severe chill, lasting half an hour, and then intense headache, muscular pain, purging, and vomiting of bile. His temperature rose to 103°F., and during the remainder of the day, and in the night following, nausea and vomiting continued, with headache, thirst, and marked prostration. Later symptoms included a diffuse rash, herpes, and in the urine granular, hyaline, and epithelial casts. The reaction subsided gradually. The leukocytes rose to 44,050 on the 3rd day, and the opsonic index reached 2.3 on the 2nd day, returning to normal by the 5th day.

Sophian and Black[11] studied agglutination and complement fixation in serum specimens from ten students who had been vaccinated with two or three doses of a monovalent vaccine. The doses given were 500 or 1,000 million, 1,000 or 2,000 million, and 2,000 million cocci, at 7 day intervals. Their vaccine had been heated to 50°C. for 1 hour. Following the vaccinations they noted malaise, frontal headache, and slight fever with occasionally more severe symptoms; intense frontal or vertical headache, general bodily pain, nausea, vomiting, and a rise of temperature to 102–104°F. Labial herpes was seen. Using a readily agglutinable organism, they found the agglutinin titers of the sera of their vaccinated subjects to range from 1:200 to 1: 1,500. Complement was fixed in serum dilutions up to 1: 250.[12] Complement-fixing antibodies were found in low dilutions of the serum of seven of these men after an interval of 2 years.

Sophian and Black refer to Hall's experience in Kansas City in the vaccination of about 280 persons in families in which meningitis had occurred. A number of doctors and nurses were likewise inoculated, and in no instance did the disease occur subsequent to vaccination. About 100 persons in Dallas were vaccinated, but most of them did not complete the vaccination series. Two nurses developed epidemic meningitis some weeks after a series of two inoculations; both recovered.

---

[10] Davis, D. J., Studies in meningococcus infections, *J. Infect. Dis.*, 1907, iv, 558.

[11] Sophian, A., and Black, J., Prophylactic vaccination against epidemic meningitis, *J. Am. Med. Assn.*, 1912, lix, 527. Black, J. H., Prophylactic vaccination against epidemic meningitis, *J. Am. Med. Assn.*,1914, lxiii, 2126.

[12] Such figures must be accepted with reserve. They are far higher than laboratory experience would lead one to expect after such prophylactic doses of a meningococcus.

Recently Whitmore, Fennel, and Petersen[13] have reported an experience with a polyvalent lipovaccine in which total doses of 40,000 million or 80,000 million cocci were given subcutaneously in one or two injections. 55 men in all were vaccinated. 40,000 million cocci in one dose did not cause any general reaction. Two doses of 40,000 million cocci each at a 3 day interval were followed by two instances of constitutional reaction among 25 men. 5 men received 80,000 million cocci in a single injection which was followed after 24 hours by a moderate general reaction. In the first days after vaccination with the larger doses agglutinin formation was observed against three of the vaccine strains, especially those that respond to antibodies of both the normal and the para type.

These reports from the literature coincide with the present experience with meningococcus vaccine in their descriptions of the reactions that may be expected, and of the appearance of specific antibodies in the blood after vaccination. As Sophian and Black pointed out, the general symptoms indicate some degree of meningeal irritation and occasionally they may simulate the onset of meningitis. The symptoms are not progressive, however, and even though severe, they clear up in a few hours. In one instance in which a lumbar puncture was done on a suspect 3 days after a second dose of vaccine the spinal fluid was found normal. The illness described by Davis is instructive to show the severity of the symptoms which may follow an injection of meningococcus substance many times the proper dose. Whitmore, Fennel, and Petersen, by protecting their vaccine in oil, were able to give much larger doses in a single injection with only moderate constitutional effects.

Whatever may be the relation of agglutinins to specific protection against invasion the agglutination test is recognized as the most reliable indication of antibody formation due to the meningococcus, and is used generally in the standardization of therapeutic sera as an index of potency. With equal reason the presence of agglutinins may be taken as an index of active immunization after vaccination. We do not know the ratio of agglutinin formation to protective power, and can only discover by wide experience what agglutinin titers correspond to relatively complete immunity to meningococcus invasion. It is perhaps significant, however, that the agglutinin titers

[13] Whitmore, E. R., Fennel, E. A., and Petersen, W. F., An experimental investigation of lipovaccines: a preliminary note, *J. Am. Med. Assn.*, 1918, lxx, 427.

of the sera of vaccinated men are of the same order of serum dilutions, namely 1 : 4 to 1 : 64+, as those of chronic carriers of the meningococcus, who are usually refractory to the strains carried. A study of the blood of cases of meningitis which have recovered without serum treatment might be instructive in this connection.

Meningococcus agglutinins appear not to have been found previously in the blood of chronic carriers. Cathoire,[14] in a brief communication, reported that the agglutinin study of carrier sera led to no positive result, but with the Wright technique he was able to show a constant increase in opsonic power compared with sera from normal persons, and he therefore concluded that the relative immunity of carriers is to be explained by a specific change in the serum.

Herrick[15] thinks that the occurrence of relapses in some cases of epidemic meningitis "lends discouragement to vaccine prophylaxis and other measures for the production of immunity." It should be pointed out that relapses are probably caused by reinfection from small pockets in the meninges in which the meningococcus has been walled off, and so permitted to survive therapeutic measures otherwise effective. It may be mentioned in passing that the occurrence of relapses in typhoid fever has been no contraindication to the employment of typhoid prophylaxis. The object of prophylactic vaccination is to oppose the meningococcus at the threshold, and if a systemic invasion precedes the spinal infection, as recent observations tend to show, the building up of antibodies in the blood stream is the means by which a hematogenous incursion is to be combated.

SUMMARY.

1. A meningococcus vaccine suspended in salt solution has been given subcutaneously as a prophylactic to about 3,700 volunteers in three injections of 2,000 million, 4,000 million, and 4,000 or 8,000 million cocci at weekly intervals.

2. These doses rarely caused more than the mildest local and general reactions. Exceptionally a more severe reaction emphasized the

[14] Cathoire, E., Recherche du pouvoir opsonisant du sérum des porteurs sains de méningocoques, *Compt. rend. Soc. biol.*, 1910, lxix, 240.

[15] Herrick, W. W., The intravenous serum treatment of epidemic cerebrospinal meningitis, *Arch. Int. Med.*, 1918, xxi, 541.

presence of an unusual individual susceptibility to the vaccine. In such instances the symptoms were in part those of meningeal irritation and sometimes simulated the onset of meningitis.

3. Specific meningococcus agglutinins have been demonstrated in the blood serum of vaccinated men as compared with normal controls.

4. Moreover, agglutinins have been demonstrated in the blood serum of chronic carriers of the meningococcus. Evidence is thus brought forward that the relative immunity of chronic carriers to epidemic meningitis may be due to the presence of specific antibodies in the blood stream.

EXPLANATION OF PLATES.

PLATE 47.

FIG. 1. Capsule of glass tubing of 2 mm. internal diameter showing points at which it is snapped off for use, and file mark for measuring equal volumes of serum and meningococcus suspension.

FIG. 2. Appearance of agglutination specimens after incubation. (*a*) Complete agglutination. (*b*) No agglutination. (*c*) Partial agglutination.

PLATE 48.

FIG. 3. Appearance of completely agglutinated specimens after shaking.

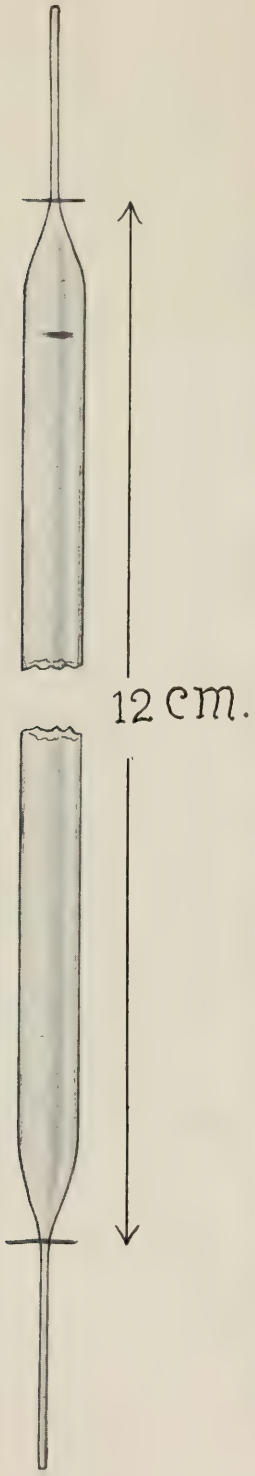


FIG. 1.

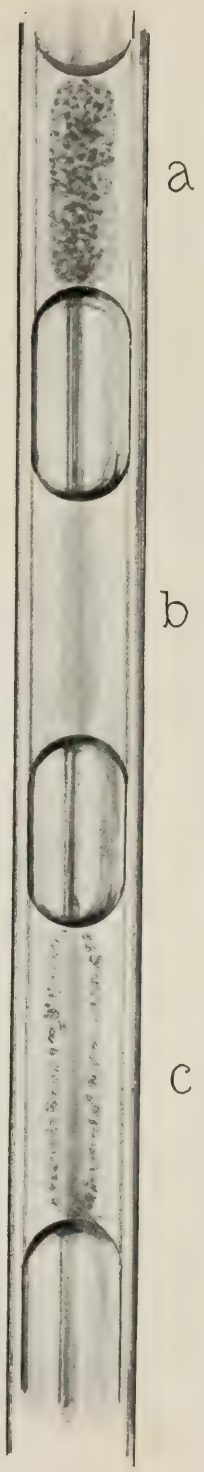


FIG. 2.

(Gates: Antimeningitis vaccination.)

FIG. 3.

(Gates: Antimeningitis vaccination.)

[Reprinted from The Journal of Experimental Medicine, October 1, 1918, Vol. xxviii, No. 4, pp. 501–516.]

THE STABILITY OF THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN NATURALLY NEPHROPATHIC ANIMALS AND THE EFFECT ON RENAL FUNCTION OF CHANGES IN THIS EQUILIBRIUM.

I. A Study of the Acid-Base Equilibrium of the Blood in Naturally Nephropathic Animals and of the Functional Capacity of the Kidney in Such Animals Following an Anesthetic.*

By WILLIAM deB. MacNIDER, M.D.

(From the Laboratory of Pharmacology of the University of North Carolina, Chapel Hill.)

Plates 49 and 50.

(Received for publication, May 31, 1918.)

As a result of the observations of Ophüls,[1] Pearce,[2] and Dayton,[3] the fact is generally known that many of the lower animals, particularly the dog, are susceptible to a type of kidney injury which should be classed as a chronic nephropathy. In a recent study[4] of the naturally acquired chronic nephropathy of the dog these earlier observations have been confirmed, the various nephropathic processes have been classified, and a consideration of the processes of repair in the kidneys has been undertaken. In this study of forty-two naturally nephropathic animals I found it possible, with three exceptions, to classify the kidney injury as a chronic productive type. The three remaining animals showed the typical arteriosclerotic type of kidney with extensive general sclerosis of the vessels. The thoracic aorta in one of the animals was the seat of a fusiform aneurysm. In the majority of kidneys of the remaining thirty-nine animals the

* Aided by a grant from The Rockefeller Institute for Medical Research.

[1] Ophüls, W., Some interesting points in regard to experimental chronic nephritis, *J. Med. Research*, 1908, xviii, 497.

[2] Pearce, R. M., An experimental study of nephrotoxins, *Univ. Penn. Med. Bull.*, 1903–04, xvi, 217.

[3] Dayton, H., Reliability of dogs as subjects for experimental nephritis, *J. Med. Research*, 1914–15, xxxi, 177.

[4] MacNider, W. deB., A pathological and physiological study of the naturally acquired chronic nephropathy of the dog. Part I, *J. Med. Research*, 1916, xxxiv, 177.

formation of connective tissue was a focal process confined to the glomeruli. In all the animals both the capsule and capillaries of the glomeruli participated in the laying down of connective tissue so that in the different animals it was not possible to specialize the glomerular pathology into a capsular and intracapillary glomerulonephropathy. Hyaline degeneration of the fibrosed capillary tufts was occasionally observed.

The formation of intertubular connective tissue in the kidneys of these animals has shown no parallel with the degree of fibrosis which has taken place in the glomeruli, and, furthermore, there has existed a notable disproportion between the severity of the changes in the glomeruli and the degree of degeneration of the tubular epithelium. This observation has been recently confirmed by Stengel, Austin, and Jonas[5] in a study of the chronic nephropathies in human material.

The above outline of the naturally acquired chronic nephropathy of the dog not only establishes the frequency of the occurrence of such conditions in these animals but shows the close histological resemblance between certain nephropathies of the lower animals and man.

The ability to obtain such material for experimental purposes offers many possibilities for the study of the chronic nephropathies and for the study of various acute processes which may be superimposed upon the naturally acquired chronic kidney injury.

The first study[6] of this character has consisted in an investigation of the functional response of the naturally nephropathic kidney after the kidney had been acutely injured by uranium nitrate or by an anesthetic. An analysis of these experiments shows that when a naturally nephropathic animal is anesthetized by Gréhant's anesthetic, or when the animal is given uranium and anesthetized by ether, the animals fall into two clearly defined groups. One group of animals during the anesthesia becomes rapidly anuric and fails to show a functional response to such diuretic substances as theobromine, caffeine, and solutions of urea and glucose. The second group of animals remains diuretic following the anesthetic and shows a functional response to the diuretic substances which in the first group of animals were of no diuretic value.

A physiological study of the response of the vascular mechanism of the kidney in the anuric and diuretic groups of animals by the use of such peripherally acting stimuli as the members of the caffeine group and adrenalin has shown this

---

[5] Stengel, A., Austin, J. H., and Jonas, L., A comparison of the functional and anatomic findings in a series of cases of renal disease, *Arch. Int. Med.*, 1918, xxi, 313.

[6] MacNider, A pathological study of the naturally nephropathic kidney of the dog, rendered acutely nephropathic by uranium or by an anesthetic. Part II, *J. Med. Research*, 1916, xxxiv, 199.

mechanism to be responsive in both groups of animals. The degree of vasodilation or constriction of the renal vessels induced by caffeine or adrenalin was usually greater in the anuric than in the diuretic groups of animals. Renal vasodilation effected by caffeine, theobromine, or solutions of urea or glucose in the diuretic group of animals was associated with a free diuresis, while with the production of an even greater degree of vasodilation by these substances in the anuric animals no formation of urine was induced.

The histological study of the kidneys of these two groups of animals has shown a chronic productive nephropathy in which the changes were largely confined to the glomeruli. As a result of the use of uranium or an anesthetic no acute degenerative changes had developed in the glomeruli. The vascular pathology in the two groups has shown no elements of difference. The acutely developing pathological change which differentiates the anuric from the diuretic groups of animals has consisted in the degree of degeneration occurring in the tubular epithelium, and especially in the epithelium of the convoluted tubules. The animals which have remained diuretic and responsive to diuretic substances have shown but slight epithelial damage, while the group of animals that have been rendered anuric by the anesthetic and non-responsive to the same diuretic substances have shown a swelling and necrosis of the convoluted tubule epithelium. The ascending limbs of Henle's loops have contained a large amount of stainable fat.

The following investigation has been undertaken with the object in view of ascertaining the difference in the response of the normal and naturally nephropathic kidney to Gréhant's anesthetic, the principal anesthetic ingredient of which is chloroform. The study embraces an investigation of the acid-base equilibrium of the blood in these two groups of animals, prior to and during the period of anesthesia, and the association of the changes in this equilibrium with the development of an anuria. The functional capacity of the kidney has been determined by the phenolsulfonephthalein test, the retention of blood urea, and the response of the kidney during the period of anesthesia to various diuretic substances. Finally, the relative toxicity of this anesthetic for the normal as compared with the naturally nephropathic kidney has been investigated by a histological study of the kidneys at the termination of the experiments.

EXPERIMENTAL.

Dogs were employed in the experiments which furnish the basis for this study. Nine of the animals were healthy dogs varying in age from 8 months to 6 years and 2 months. Eighteen of the dogs were naturally nephropathic and varied in age from 3 years to 13 years and 1 month. Both the normal animals which served as controls and the naturally nephropathic animals were placed in metabolism cages, given 500 cc. of water daily, and fed on bread with a small amount of cooked meat. The animals were studied for 3 days prior to the day of experiment. During this period the urine was collected twice a day and examined qualitatively for albumin, glucose, acetone, and diacetic acid. Quantitative determinations of these substances were made when present. The centrifugalized urine was examined for casts. The hydrogen ion content of the blood was determined by the method of Levy, Rowntree, and Marriott,[7] the alkali reserve of the blood and the tension of carbon dioxide in alveolar air by the methods of Marriott,[8,9] while the blood urea was determined by the method of Marshall[10] as modified by Van Slyke and Cullen.[11] The phenolsulfonephthalein test for kidney function was conducted according to the technique of Rowntree and Geraghty.[12]

At the end of the 3 day period allowed for normal observations the animals were given 300 cc. of water and 3 hours later were given 60 cc. per kilo of Gréhant's anesthetic by stomach tube. Half an hour was allowed for the development of a degree of anesthesia sufficient for

[7] Levy, R. L., Rowntree, L. G., and Marriott, W. McK., A simple method for determining variations in the hydrogen-ion concentration of the blood, *Arch. Int. Med.*, 1915, xvi, 389.

[8] Marriott, W. McK., A method for the determination of the alkali reserve of the blood plasma, *Arch. Int. Med.*, 1916, xvii, 840.

[9] Marriott, The determination of alveolar carbon dioxid tension by a simple method, *J. Am. Med. Assn.*, 1916, lxvi, 1594.

[10] Marshall, E. K., Jr., A rapid clinical method for the estimation of urea in urine, *J. Biol. Chem.*, 1913, xiv, 283.

[11] Van Slyke, D. D., and Cullen, G. E., A permanent preparation of urease, and its use in the determination of urea, *J. Biol. Chem.*, 1914, xix, 211.

[12] Rowntree, L. G., and Geraghty, J. T., An experimental and clinical study of the functional activity of the kidneys by means of phenolsulphonephthalein, *J. Pharmacol. and Exp. Therap.*, 1909–10, i, 579.

the surgical part of the experiment. The first observations were made on the anesthetized animals 1 hour after they had received the anesthetic and half an hour after the development of a satisfactory state of general anesthesia. At half hour periods during the course of the experiments the flow of urine per minute was recorded and the hydrogen ion content and reserve alkali of the blood were determined. At these intervals the animals were given intravenously one of the following diuretic substances: caffeine citrate or theobromine sodium salicylate in 1 per cent solution, 1 cc. per kilo; pituitrin (Parke, Davis and Company) 0.5 cc.; or solutions of urea or glucose. The urea solution was of 0.9 per cent strength in 0.9 per cent sodium chloride and was given in the quantity of 10 cc. per kilo. The glucose solution was 20 per cent strength in 0.9 per cent sodium chloride and was given in the same quantity per kilo as was the urea solution.

The experiments on the anesthetized animals were terminated at the end of $1\frac{1}{2}$ hours at which time the tension of carbon dioxide in alveolar air was determined and the kidneys were removed for the histological study.

Observations on Normal and Naturally Nephropathic Animals Prior to an Anesthetic.

The following observations which have extended over a period of 3 days have been recorded in Table I. In this table observations on two normal or control animals have been tabulated with similar observations on nine of the naturally nephropathic animals.

In recent papers[13, 14] studies have been made of the relative stability of the acid-base equilibrium of the blood in animals of different ages. In these studies it was found that the older animals were more susceptible to agents which altered this equilibrium in favor of the acid ion than were the younger animals. For this reason relatively young animals were selected for the controls in these experiments. The factor of the age of the animal as expressed by the ease with which

[13] MacNider, A consideration of the relative toxicity of uranium nitrate for animals of different ages. I, *J. Exp. Med.*, 1917, xxvi, 1.

[14] MacNider, Concerning the influence of the age of an organism in maintaining its acid-base equilibrium, *Science*, 1917, xlvi, 643.

the nephropathic animals develop an acid intoxication cannot be accurately determined in these experiments, since the existence of the chronic nephropathy in the animals, as will be demonstrated later in this study, makes them more susceptible to changes in the acid-base equilibrium. The naturally nephropathic animals have, however, been arranged in the table according to their age. The influence of this factor in determining the toxicity of the anesthetic will be referred to in a later part of this paper.

Reference to Table I shows the control animals to be freely diuretic and the urine to be free from albumin, glucose, acetone bodies, and casts. The hydrogen ion content of the blood has varied from 7.3 to 7.45, while the reserve alkali has shown a variation from 8 to 8.1. The hydrogen ion determinations by the method employed in these experiments do not show a correlation with the reserve alkali determinations or the determinations of alveolar air carbon dioxide. The Levy-Rowntree-Marriott method is an expression of both the volatile and non-volatile acid content of the blood. Even a local accumulation of carbon dioxide in the blood of a part, such as the arm or leg, may give a very high reading and is not a true expression of the non-volatile acid content of the blood. For this reason the alkali reserve determinations and the variations in alveolar air carbon dioxide tension more accurately indicate the changes in the hydrogen ion content.

The determinations of alveolar air carbon dioxide for the normal animals have varied between 37 and 40 mm. and have shown a correlation with the maximum and minimum variations of the reserve alkali of the blood.

The blood urea estimations have remained very constant. In one of the nine control animals the percentage of urea was 0.014 per cent, while in the remaining animals the blood urea was 0.012 per cent. The phenolsulfonephthalein test was made on six of the control animals. The total output of the dye in a 2 hour period varied from a minimum of 73 per cent to a maximum of 81 per cent.

A study of the observations on the naturally nephropathic animals prior to an anesthetic (Table I) shows a marked variation in the output of urine by the different animals in a 24 hour period. This has varied in the respective animals from 174 to 820 cc. The urine of all the animals contained albumin and casts. In all but two of the animals

TABLE I.

Observations on Normal and Naturally Nephropathic Animals Prior to an Anesthetic.

Experiment No.	Age of animal.	Weight.	Water in 24 hrs.	Urine in 24 hrs.	Albumin and casts.	Acetone and diacetic acid.	pH	R. pH	Carbon dioxide.	Blood urea.	Phenolsulfonephthalein.
	yrs.	*kg.*	*cc.*	*cc.*					*mm.*	*per cent*	*per cent*
1 (control).	1	8.2	500	481	0	0	7.3	8.0	37	0.012	
2 (control).	3	13.66	500	287	0	0	7.45	8.1	40	0.012	81
3	1–2	11.3	500	174	Tr. Casts.	0	7.45	8.15	40	0.015	68
4	1–2	21.53	500	518	" "	0	7.45	8.1	39		
5	1–2	7.32	500	389	1.2 gm. Numerous casts.	0	7.45	7.9	36	0.018	54
6	3 yrs. and 4 mos.	11.4	500	584	Tr. Casts.	0	7.4	8.0	40		
7	8	17.7	500	820	" "	0	7.45	8.1	40		
8	10	23.1	500	508	" "	0	7.45	8.0	38		
9	10–11	21.55	500	355	" Numerous casts.	0	7.35	8.0	38	0.015	61
10	12	23.4	500	718	" Few casts.	0	7.45	8.0	41	0.015	69
11	13 yrs. and 1 mo.	30.35	500	520	0.9 gm. Few casts.	0	7.45	8.1	32	0.028	52

albumin was present as a mere trace. In one animal the urine contained 1.2 gm. of albumin per liter. This animal (Experiment 5, Table I) as contrasted with the other naturally nephropathic animals showed a depletion in the alkali reserve of the blood, a decrease in the tension of alveolar air carbon dioxide, and a marked reduction in the elimination of phenolsulfonephthalein. None of the naturally nephropathic animals of this series has shown the presence of acetone bodies in the urine. The hydrogen ion determinations have been variable as was the case with the control animals. With one exception (Experiment 5, Table I) the alkali reserve of the blood and the tension of alveolar air carbon dioxide in the naturally nephropathic animals have been within the range of normality. The reserve alkali in these animals has varied from 8 to 8.1, while the carbon dioxide tension has varied between 32 and 41 mm. This minimum variation of 32 mm. in carbon dioxide tension does not correlate with the reserve alkali reading for the blood which was 8.1. The question arises as to whether or not some local pathology in the lung of this very old animal could not have been responsible for this atypical reading.

The blood urea determinations have shown a retention in all the naturally nephropathic animals, varying from 0.015 to 0.028 per cent. The elimination of phenolsulfonephthalein has been reduced in all the animals and, as is shown in Table I, there is a relation between the retention of blood urea and the elimination of phenolsulfonephthalein. The animal of Experiment 11, with the greatest retention of blood urea, 0.029 per cent, also shows the greatest reduction in the output of phenolsulfonephthalein which was 52 per cent. The animals with a lower percentage retention of blood urea have a higher percentage elimination of the dye.

From the foregoing analysis of observations on the control and naturally nephropathic animals prior to the use of an anesthetic, the following conclusions are permissible: (1) The control animals show no evidence of a kidney injury and have a normal acid-base equilibrium. (2) The naturally nephropathic animals give evidence of a chronic kidney injury by the formation of a variable amount of urine which contains albumin and casts, by a retention of blood urea, and by a decrease in the elimination of phenolsulfonephthalein. (3) The naturally nephropathic animals with one exception, Experiment 5,

show a normal acid-base equilibrium. (4) In the naturally acquired chronic nephropathy of the dog in which the chronic pathology is largely confined to the glomeruli an acid intoxication is not the primary cause for the kidney injury. (5) In such chronic nephropathies blood urea determinations and estimations of the ability of the kidney to eliminate phenolsulfonephthalein are of more diagnostic value than determinations of the acid-base equilibrium of the blood.

Observations on Normal and Naturally Nephropathic Animals after an Anesthetic.

An analysis of the response of the control animals to Gréhant's anesthetic as indicated in Table II shows these animals to have remained diuretic following the development of a state of surgical anesthesia. The urine flow varied between 1 and 2 drops per minute for both animals. Half an hour after the establishment of a state of anesthesia the urine flow was unaffected and no change from the normal alkali reserve reading of 8 to 8.1 had occurred. During the remaining hour of the experiment these animals were freely diuretic to theobromine, caffeine, pituitrin, and a solution of glucose. The flow of urine from pituitrin was increased from 5 to 20 drops per minute and an even greater diuretic effect was obtained from the glucose solution, the urine increasing from 5 to 26 drops per minute.

During the course of the experiments the control animals were able to maintain their normal acid-base equilibrium. The alkali reserve of the blood failed to show any depletion and the tension of carbon dioxide in alveolar air remained practically unaffected. At the end of the experiment the control animals were forming a larger amount of urine than was the case at the commencement of the anesthesia. At the commencement of the experiments the urine flow per minute for the animals of Experiments 1 and 2 was 2 and 1 drops for the respective animals, while at the termination of the experiments the urine flow was 3 and 6 drops per minute for these animals.

The histological study of the kidneys of the control animals has been negative in as far as demonstrating any pathological change induced by the anesthetic. The glomerular vessels are distended with blood and the capillary loops usually fill the capsular space. The tubular epithelium, and especially that of the convoluted tubules,

is shrunken, the nucleus-plasma relationship has increased in favor of the nucleus, and the nuclei are hyperchromatic and stain intensely. The ascending limbs of Henle's loops either contain no stainable fat or a very small amount of fat in the form of dust-like particles.

A study of the response of the naturally nephropathic animals to Gréhant's anesthetic given in the same quantity per kilo as was the case with the control animals shows (with two exceptions, Experiments 4 and 6, Table II) that all the naturally nephropathic animals were rendered anuric by the anesthetic in ½ hour after the anesthetic was administered. At this period in the experiments the table shows that the anesthetic had induced a rapid depletion in the alkali reserve of all the naturally nephropathic animals which had become acutely anuric, while in the animals which at this period remained diuretic the alkali reserve had either undergone no change from the normal reading prior to the anesthetic or the reduction in the alkali reserve was not below 8.05. For example, the naturally nephropathic animal of Experiment 4 remained diuretic following the anesthetic and showed only a slight variation in the alkali reserve reading, 8.1 to 8.05. The animal of Experiment 6, which also remained diuretic, showed no change in the alkali reserve. The reading was 8 before and after the development of an anesthesia. The naturally nephropathic animals which became rapidly anuric show a depletion in the alkali reserve. The animal of Experiment 3 showed a reduction in the reserve alkali from 8.15 to 8, while in the animal of Experiment 8 the reserve alkali was reduced by the anesthetic from a normal reading of 8 to 7.9.

At this period of the experiments, the end of the first half hour, the functional response of the kidney was investigated by giving the animals caffeine, theobromine, or pituitrin. A study of Table II shows these substances, in the animals in which the anesthetic had induced a rapid depletion of the alkali reserve of the blood, to be of no diuretic value. The animals remained anuric. In the animal of Experiment 6, in which there had occurred no change in the alkali reserve from the normal reading, theobromine induced a free diuresis, the output of urine increasing from 2 to 10 drops per minute. In the animal of Experiment 4 in which the anesthetic had brought about a reduction in the alkali reserve from 8.1 to 8.05 caffeine was of no diuretic value.

At the end of the 1st hour of the anesthesia all the naturally nephropathic animals had become anuric. The alkali reserve of the blood had been reduced to 7.9 in all the animals except the dog of Experiment 10. The reserve alkali reading for this animal was 7.8. At this period of the experiment the functional response of the kidney was again tested by employing as diuretics theobromine, or solutions of urea and glucose. The kidneys of the naturally nephropathic animals were non-responsive to these substances which in the control animals had induced a marked diuretic effect. The anuria which has been associated with the development of an acid intoxication on the part of the anesthetized naturally nephropathic animals was unaffected by these diuretic solutions.

The experiments were continued for the third half hour period. During this time the animals remained anuric and the reserve alkali of the blood showed a progressive decrease in all the animals. At the termination of the experiments, $1\frac{1}{2}$ hours after the first observations had been made, the reserve alkali readings for all the naturally nephropathic animals varied from a maximum reading of 7.85 to the extremely low reading of 7.45. The determinations of carbon dioxide tension in alveolar air at the close of the experiments varied between 22 and 10 mm. and showed the usual correlation with determinations of the alkali reserve of the blood.

In view of the previously mentioned observation that old animals were more susceptible to agents which induced an acid intoxication than were young animals, it is interesting to note that the two oldest naturally nephropathic animals gave evidence of having developed the severest acid intoxication. The animal of Experiment 10, 12 years old, had at the termination of the experiment an alkali reserve of 7.6 and a tension of alveolar air carbon dioxide of 18 mm., while the animal of Experiment 11, 13 years and 1 month old, had a reserve alkali of only 7.45 and a tension of alveolar air carbon dioxide of 10 mm.

The histological study of the kidneys of the naturally nephropathic animals after the establishment of an anuria by Gréhant's anesthetic has not shown any acute degenerative change or other evidence of vascular injury to the glomeruli. The capillary tufts usually fill the capsular space unless their distention has been prevented by a forma-

TABLE II.

Toxic Effect of an Anesthetic on the Functional Capacity of Normal and Naturally Nephropathic Kidneys.

Experiment No.	Anesthetic (Gréhant's).	Urine per min.	pH ½ hr. after anesthetic.	R. pH ½ hr. after anesthetic.	Urine per min.	Diuretic.	Urine per min.	pH 1 hr. after anesthetic.	R. pH 1 hr. after anesthetic.	Diuretic.	Urine per min.	pH 1½ hrs. after anesthetic.	R. pH 1½ hrs. after anesthetic.	Carbon dioxide 1½ hrs. after anesthetic.	Urine per min.	Fat in renal epithelium.
	per cent	*gtt.*			*gtt.*		*gtt.*				*gtt.*			*mm.*	*gtt.*	
1 (control).	60	2	7.45	8.0	2	Theobromine 1%.	5	7.45	8.0	Pituitrin 0.5 cc.	21	7.35	8.0	36	3	Tr.
2 (control).	60	1	7.4	8.1	2	Caffeine 1%.	5	7.4	8.1	Glucose sol. 20%.	26	7.4	8.1	39	6	"
3	60	0	7.45	8.0	0	Pituitrin 0.5 cc.	0	7.4	7.9	Urea sol. 0.9%.	0	7.1	7.8	19	0	L.*
4	60	2	7.4	8.05	0	Caffeine 1%.	0	7.3	7.9	" " 0.9%.	0	7.25	7.85	22	0	"
5	60	0	7.45	7.8	0	Theobromine 1%.	0	Not made.	Not made.	Glucose sol. 20%.	0	7.35	7.85	Not made.	0	"
6	60	2	7.35	8.0	2	Theobromine 1%.	10	7.4	7.9	Theobromine 1%.	0	7.25	7.85	21	0	"
7	60	0	7.3	8.0	0	Caffeine 1%.	0	7.35	7.9	Theobromine 1%.	0	7.2	7.8	20	0	"
8	60	0	7.4	7.9	0	Theobromine 1%.	0	7.4	7.9	Theobromine 1%.	0	7.3	7.85	20	0	"
9	60	0	7.2	7.9	0	Caffeine 1%.	0	7.4	7.9	Urea sol. 0.9%.	0	7.35	7.8	21	0	"
10	60	0	7.35	7.9	0	Theobromine 1%.	0	7.4	7.8	Theobromine 1%.	0	7.3	7.6	18	0	V. L.
11	60	0	7.4	8.0	0	Pituitrin 0.5 cc.	0	7.35	7.9	Urea sol. 0.9%.	0	7.15	7.45	10	0	"

*L, indicates large amount; V. L., very large amount.

tion of connective tissue. The characteristic and constant change which is induced by the anesthetic is an acute swelling, vacuolation, and necrosis of the epithelium of the convoluted tubules, and a rapid accumulation of stainable fat in the ascending limbs of Henle's loops. Fatty degeneration of a slight degree is occasionally seen in the degenerating convoluted tubule epithelium. The amount of stainable fat in the epithelium has shown a relation with the degree of acid intoxication in the various animals. The animals which have shown the greatest depletion in their alkali reserve and the lowest tension of carbon dioxide in alveolar air have also shown the greatest accumulation of fat in the degenerated epithelium, especially the epithelium of Henle's loops (Figs. 1 and 2).

DISCUSSION.

During the past 10 years numerous investigations have been concerned with the occurrence and significance of an acid intoxication in the acute and chronic nephropathies. As early as 1888 von Jaksch[15] noted a decrease in the alkalinity of the blood in uremia and this observation was confirmed in 1898 by Brandenburg.[16] Von Hösslin[17] in 1909 noted in certain of the nephropathies a definite relation between the acidity of the urine and the amount of albumin and number of casts, and in a later paper[18] after observing that very large amounts of alkali were necessary to reduce the acidity of the urine in nephritics, recommended rather indiscriminately the use of an alkali as a therapeutic measure. In 1912 Sellards,[19] employing his alkaline tolerance test in a group of nephropathies, noted a retention of bicarbonate in the acute nephropathies with uremia, and at

---

[15] von Jaksch, R., Ueber die Alkalescenz des Blutes bei Krankheiten, *Z. klin. Med.*, 1888, xiii, 350.

[16] Brandenburg, K., Ueber die Alkalescenz des Blutes, *Z. klin. Med.*, 1899, xxxvi, 267.

[17] von Hösslin, R., Ueber die Abhängigkeit der Albuminurie vom Säuregehalt des Urins, *Münch. med. Woch.*, 1909, lvi, 1673.

[18] von Hösslin, Über die Abhängigkeit der Albuminurie vom Säuregrad des Urins und über den Einfluss der Alkalizufuhr auf Acidität, Albuminurie, Diurese und Chloridausscheidung, sowie auf das Harnammoniak, *Deutsch. Arch. klin. Med.*, 1912, cv, 147.

[19] Sellards, A. W., The determination of the equilibrium in the human body between acids and bases with especial reference to acidosis and nephropathies, *Bull. Johns Hopkins Hosp.*, 1912, xxiii, 289.

about the same time Porges and Leimdörfer,[20] using determinations of carbon dioxide tension as an index of an acid intoxication, concluded that there occurred in general a reduction of carbon dioxide tension parallel to the symptoms of uremia. The work of Straub and Schlayer[21] which was confirmed by Barcroft[22] and his pupils has apparently established the fact that in the type of acute kidney injury characterized by the symptom complex uremia, there is a direct connection between the uremic manifestations and an acid intoxication.

The question which is still undecided is concerned with the association of an acid intoxication in those acute and chronic nephropathies which have not developed symptoms of uremia and whether or not when such an intoxication occurs in these cases it should be considered as a retention acidosis due to the kidney injury or whether it should be looked upon as the cause of the renal injury. Both Sellards[23] and Peabody[24] in their studies of the chronic nephropathies reach the conclusion that the acid intoxication developing in these conditions is a retention acidosis and that the accumulation of non-volatile acids is not responsible for the kidney injury.

An analysis of the experiments which have been presented in the present study show that animals may have a severe type of chronic kidney injury that is largely localized in the glomeruli without developing an acid intoxication which can be detected by a depletion in the alkali reserve of the blood or by a reduction in the tension of alveolar air carbon dioxide. These animals show a slight retention of blood urea and a moderate reduction in the output of phenolsulfonephthalein. The minimum output of the dye in a 2 hour period for the naturally nephropathic animals has been 52 per cent. The kidneys of these animals show an epithelial element which is well preserved histologically and does not show any acute degenerative change. When these animals are anesthetized their response to the anesthetic as compared with normal animals shows the acid-base equilibrium of the naturally

[20] Porges, O., and Leimdörfer, A., Ueber die Kohlensäurespannung des Blutes in pathologischen Zuständen. III Mitteilung. Ueber die Kohlensäurespannung bei Nierenerkrankungen, *Z. klin. Med.*, 1913, lxxvii, 464.

[21] Straub, H., and Schlayer, Die Urämie eine Säurevergiftung, *Münch. med. Woch.*, 1912, lix, 569.

[22] Barcroft, J., The respiratory function of the blood, Cambridge, 1914.

[23] Sellards, A. W., The essential features of acidosis and their occurrence in chronic renal disease, *Bull. Johns Hopkins Hosp.*, 1914, xxv, 141.

[24] Peabody, F. W., Clinical studies on the respiration. II. The acidosis of chronic nephritis, *Arch. Int. Med.*, 1915, xvi, 955.

nephropathic animals to be clearly unstable, for these animals rapidly develop an acid intoxication while the control animals maintain their normal acid-base equilibrium. Furthermore, when the acid-base equilibrium of these naturally nephropathic animals is only slightly altered in the direction of an accumulation of acid ions the animals become anuric and fail to respond to a variety of diuretic substances. In the control animals which are able to maintain their normal acid-base equilibrium during the period of anesthesia these diuretic substances induce a marked increase in the formation of urine. The development of the anuria by the nephropathic animals during the period of anesthesia which coincides with the occurrence of the acid intoxication has been constantly associated with an acute degeneration of the convoluted tubule epithelium and without the development of any acute injury to the vascular tissue of the kidney.

From these experiments it would appear that in the naturally acquired kidney injury of the dog in which the chronic pathology is largely confined to the glomeruli, the injury is not due to an acid intoxication. The experiments furthermore show that when such a kidney is subjected to an agent which leads to the formation and accumulation in the blood of acid bodies, the epithelium rapidly degenerates, and that with this degeneration the functional capacity of the kidney is arrested.

CONCLUSIONS.

1. The naturally acquired chronic glomerulonephropathies of the dog are not due to an acid intoxication.

2. Such an injury renders the acid-base equilibrium of the animal unstable and susceptible to an agent such as an anesthetic which tends to induce an acid intoxication.

3. When naturally nephropathic animals are anesthetized by Gréhant's anesthetic, the principal anesthetic ingredient of which is chloroform, the animals develop an acid intoxication, and become anuric and non-responsive to diuretic substances.

4. The development of the anuria has been constantly associated with swelling, vacuolation, and necrosis of the convoluted tubule epithelium.

5. In the kidneys of these animals there occurs an accumulation of fat which is largely confined to the ascending limbs of Henle's loops and which shows a quantitative relation with the degree of acid intoxication.

EXPLANATION OF PLATES.

PLATE 49.

FIG. 1. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the naturally nephropathic animal of Experiment 5, Table II. The glomerulus, *a*, has been to a large extent converted into a mass of connective tissue. The capillaries of the glomerulus have become obliterated and adherent to the thickened capsule. Surrounding the capsule is an area, *b*, of periglomerular fibrosis. At *c* is shown the acutely swollen and vacuolated convoluted tubule epithelium which is becoming necrotic. This animal after becoming anesthetized developed an acute acid intoxication, became anuric, and failed to show any diuretic effect from theobromine or a solution of glucose.

PLATE 50.

FIG. 2. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the naturally nephropathic animal of Experiment 11, Table II. The glomeruli at *a* show both a capsular and an intracapillary formation of connective tissue. The smaller of the two glomeruli has undergone a partial hyaline degeneration. At *b* the thickening of the capsule is marked. At *c* is shown the acutely swollen convoluted tubule epithelium which is beginning to undergo necrosis. Very early during the anesthesia this animal developed an acute acid intoxication became anuric, and failed to show any diuretic effect from either pituitrin or theobromine.

FIG. 1.

(MacNider: Nephropathic animals. I.)

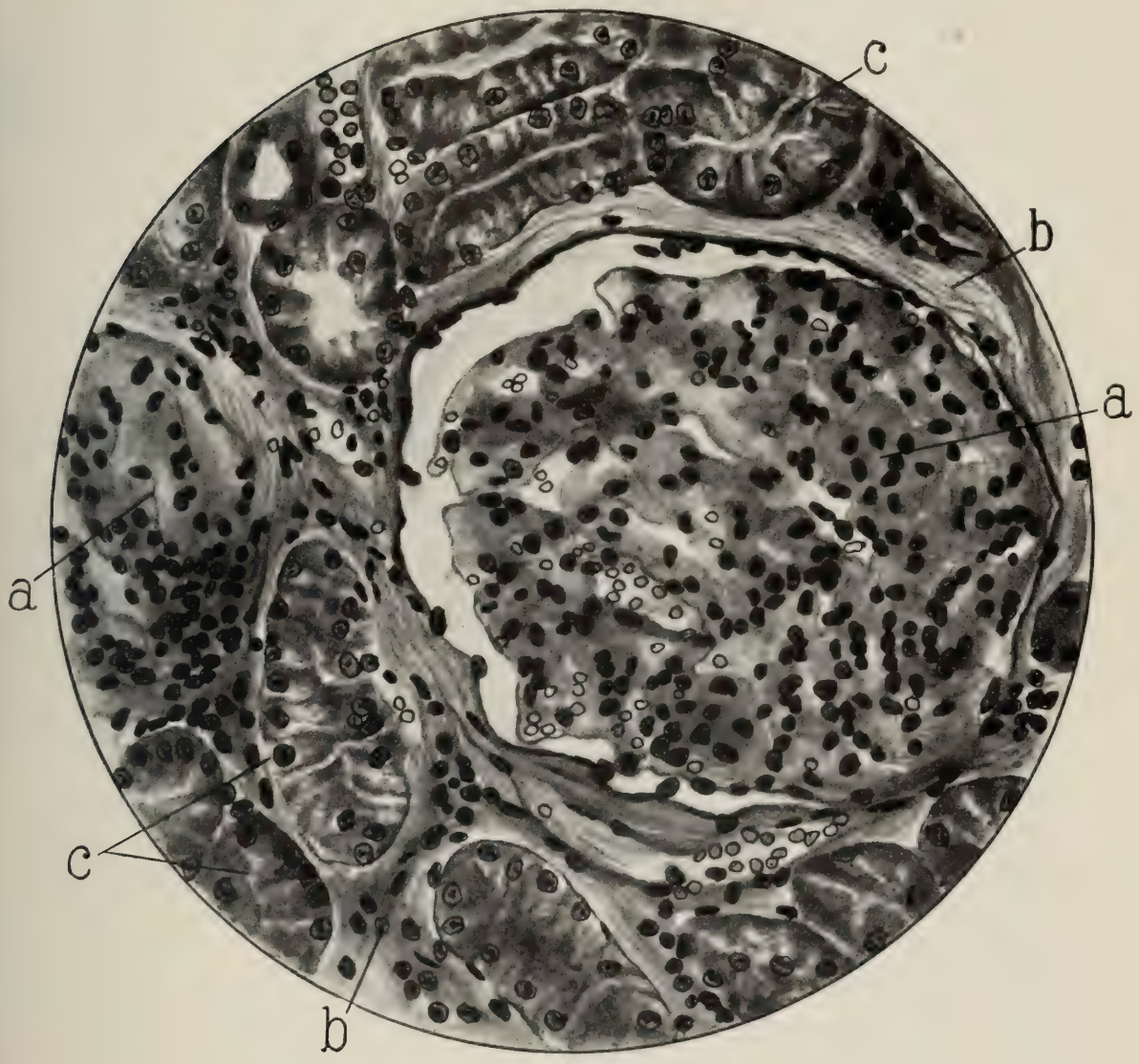


FIG. 2.

(MacNider: Nephropathic animals. I.)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, October 1, 1918, Vol. xxviii, No. 4, pp. 517–528.]

THE STABILITY OF THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN NATURALLY NEPHROPATHIC ANIMALS AND THE EFFECT ON RENAL FUNCTION OF CHANGES IN THIS EQUILIBRIUM.

II. A STUDY OF THE EFFICIENCY OF AN ALKALI TO PROTECT THE NATURALLY NEPHROPATHIC KIDNEY AGAINST THE TOXIC EFFECT OF AN ANESTHETIC.*

BY WILLIAM DEB. MACNIDER, M.D.

(*From the Laboratory of Pharmacology of the University of North Carolina, Chapel Hill.*)

PLATES 51 TO 53.

(Received for publication, May 31, 1918.)

In Part I of the present investigation, which was concerned with a study of the acid-base equilibrium of the blood in naturally nephropathic animals during the course of an anesthesia and also with the functional capacity of the kidneys of these animals, there was shown to be a relation between the depletion of the blood of its alkali reserve with the functional response of the kidney to various diuretic substances and to the development of an anuria. Naturally nephropathic animals when contrasted with normal animals as controls were shown to have an unstable acid-base equilibrium, and when the mechanism which controls this equilibrium was subjected to the action of an agent such as chloroform which tends to induce an acid intoxication, the naturally nephropathic animals very rapidly developed such an intoxication, and became anuric. The normal animals gave no evidence of an acid intoxication and in these animals the formation of urine was unaffected by the anesthetic.

* Aided by a grant from The Rockefeller Institute for Medical Research.

In two recent papers,[1, 2] which were concerned with the acute nephropathy induced in the dog by uranium I have been able to show not only an association between the degree of kidney injury and the severity of the acid intoxication induced by this metal, but have also shown that the intravenous use of an alkali in these animals would protect the kidney against the toxic effect of uranium and increase the efficiency of various diuretic substances. The first of these observations has been recently confirmed by the work of Goto.[3]

In the present study, Part II, an investigation will be made of the ability of an alkali to protect the naturally nephropathic kidney against Gréhant's anesthetic and to ascertain whether or not a sufficient degree of protection is obtained to enable the kidney to retain its responsiveness to diuretic solutions.

EXPERIMENTAL.

In this study a technique similar to that employed in Part I has been followed. Twenty-eight naturally nephropathic animals have been used in the investigation. Ten of these animals were used as control experiments, while the remaining eighteen animals were given an alkaline solution and furnished the basis for the deductions concerning the ability of an alkali to protect the kidney against the toxic effect of an anesthetic.

Observations on Naturally Nephropathic Animals Prior to Protecting the Kidney against the Anesthetic by Sodium Carbonate.

The observations on both groups of animals prior to an anesthetic are included in Table I. In this table observations are recorded on

[1] MacNider, W. deB., The inhibition of the toxicity of uranium nitrate by sodium carbonate, and the protection of the kidney acutely nephropathic from uranium from the toxic action of an anesthetic by sodium carbonate, *J. Exp. Med.*, 1916, xxiii, 171.

[2] MacNider, The efficiency of various diuretics in the acutely nephropathic kidney, protected and unprotected by sodium carbonate. II, *J. Exp. Med.*, 1917, xxvi, 19.

[3] Goto, K., A study of the acidosis, blood urea, and plasma chlorides in uranium nephritis in the dog, and of the protective action of sodium bicarbonate, *J. Exp. Med.*, 1917, xxv, 693.

two of the control animals, Experiments 1 and 2, and four of the animals which received the alkaline injection, Experiments 3 to 6. The table shows all the animals to be freely diuretic on the final day of observation prior to the use of an anesthetic. All the animals were naturally nephropathic. The urine contained albumin which varied in amount from a mere trace to 1.5 gm. per liter. Casts were present in the urine from all the animals. The urine of only one animal, Experiment 4, showed the presence of acetone bodies. The urine from this animal contained both acetone and diacetic acid. The hydrogen ion content of the blood has varied from 7.35 to 7.5. As indicated in Part I of these studies the readings do not give a true expression of the non-volatile acid content of the blood. The reserve alkali of the blood in these naturally nephropathic animals has varied between a minimum of 8 and a maximum of 8.1. The readings correlate with the determinations of alveolar air carbon dioxide which show a variation from 39 to 42 mm. These observations substantiate the conclusion made in the previous study, that the naturally nephropathic dog with kidney changes largely confined to the glomeruli does not show a depletion in the alkali reserve of the blood.

The renal function test in these animals shows a reduction in the output of phenolsulfonephthalein. The percentage elimination in a 2 hour period has varied from 54 to 64 per cent. In all the animals there is an increase in the percentage of blood urea. The animal with the highest percentage of blood urea has shown the greatest reduction in the elimination of phenolsulfonephthalein (Experiment 6, Table I). The foregoing account of observations made on naturally nephropathic animals used in this study, confirms in detail the observations made on the naturally nephropathic animals which were employed in Part I.

On the day of experiment the animals were given 300 cc. of water by stomach tube. 3 hours later under local anesthesia from a 2 per cent solution of cocaine the control animals were given intravenously 25 cc. per kilo of 0.9 per cent sodium chloride solution; while the animals which were to receive the protection against the anesthetic were given intravenously 25 cc. per kilo of a solution of sodium carbonate equimolecular with 0.9 per cent sodium chloride. The animals were then anesthetized by Gréhant's anesthetic in 60 per

TABLE I.

Observations on Naturally Nephropathic Animals Prior to Protecting the Kidney against the Anesthetic by Sodium Carbonate.

Experiment No.	Age of animal.	Weight.	Water in 24 hrs.	Urine on day of experiment.	Albumin and casts.	Acetone per 100 cc.	Diacetic acid per 100 cc.	pH	R. pH	Carbon dioxide.	Blood urea.	Phenol-sulfone-phthalein.
	yrs.	*kg.*		*cc.*		*mg.*	*mg.*			*mm.*	*per cent*	*per cent*
1 (control).	6	12.4	500	498	Tr. Few casts.	0	0	7.45	8.0	40	0.014	34
2 (control).	10 yrs. and 3 mos.	17.4	500	835	0.9 gm. " "	0	0	7.5	8.1	42	0.016	58
3	6	15.65	500	478	Tr. " "	0	0	7.35	8.1	40	0.015	56
4	11	17.4	500	508	" " "	11.3139	3.6456	7.4	8.0	39	0.018	64
5	13 yrs. and 2 mos.	7.85	500	611	1.5 gm. " "	0	0	7.4	8.05	40	Not made.	
6	15	18.9	500	515	Tr. " "	0	0	7.5	8.0	38	0.028	51

cent strength. 1 hour after giving the anesthetic the first observations were made on the acid-base equilibrium of the blood, the formation of urine, and the response of the kidney to various diuretic substances. The details of these observations are recorded in Table II.

A study of the control animals of Table II which received prior to the anesthetic a solution of sodium chloride shows a response on the part of these animals to the anesthetic similar to that obtained with all the naturally nephropathic animals used in Part I of these studies. The animals show a rapid depletion in the alkali reserve of the blood so that by the end of the 1st hour of the experiments the reserve alkali readings have been reduced to 7.9. At this stage of the experiments both the control animals, Experiments 1 and 2, had become anuric. The animal of Experiment 1 was given a solution of theobromine, while the second control animal, Experiment 2, was given pituitrin. The animals remained anuric and non-responsive to these diuretic substances. Half an hour after giving these solutions the alkali reserve of the blood in both the control animals was reduced to 7.85. During this period no urine had been formed. 2 hours after the commencement of the anesthesia the control animal of Experiment 1 was given 10 cc. per kilo of a 0.9 per cent solution of urea, while the second control animal was given a 1 per cent solution of theobromine. To these diuretic solutions there was no response. The blood at this stage of the experiments showed a further depletion of reserve alkali. The alkali reserve reading for the animal of Experiment 1 was 7.7, while the reading for the second control animal, Experiment 2, was 7.6. The tension of alveolar air carbon dioxide showed a proportionate reduction. The tension in Experiment 1 was reduced to 19 mm., while in Experiment 2 the tension was reduced to 15 mm. At this stage of the experiments, 2 hours after the commencement of the anesthesia, the kidneys were removed and the experiments terminated.

The histological study of the kidneys of these naturally nephropathic animals which received a solution of sodium chloride and served as control experiments shows changes similar in character to those described for the naturally nephropathic animals of Part I. The kidneys show a chronic glomerulonephropathy. The acute changes which have been induced in the kidneys by the anesthetic and which have been associated with the development of an acid intoxication

and an anuria, consist in an acute swelling and necrosis of the convoluted tubule epithelium and the deposition of large amounts of stainable fat in the ascending limbs of Henle's loops.

The following conclusions are permissible from the observations on naturally nephropathic animals which have served as control experiments: (1) A 0.9 per cent solution of sodium chloride given to a naturally nephropathic animal prior to an anesthetic has no effect in protecting the animal against an acid intoxication resulting from the anesthetic. (2) With a blood hydremic from such a solution various diuretic substances as pituitrin, theobromine, and solutions of urea are ineffective as diuretics.

Protection of the Naturally Nephropathic Kidney against the Toxic Effect of an Anesthetic by Sodium Carbonate.

A study of the animals of Table II, Experiments 3 to 6, which received solutions of sodium carbonate, shows the effect of such solutions on the acid-base equilibrium of the blood of naturally nephropathic animals and the efficiency of the solution in protecting the kidney against the toxic effect of the anesthetic. These experiments when compared with the control animals demonstrate that the use of the carbonate solution conferred sufficient protection against the anesthetic to prevent the animals from becoming anuric during the development of an anesthesia. As will be seen from a study of Table II, all the control animals at this early period of the anesthesia had become anuric.

1 hour after the commencement of the anesthetic the carbonate animals continued to remain diuretic, the flow of urine in the respective animals varying between one to two drops per minute. The alkali reserve of the blood at this period of observation as a result of the use of the carbonate solution has shown an increase in alkali above the normal. The readings vary from 8.25 to 8.3. With the alkali reserve increased to this degree the animals were given either a solution of caffeine or pituitrin to test the functional response of the kidney. Two of the animals, Experiments 3 and 5, responded to these diuretics by an increased formation of urine, while the two remaining animals, Experiments 4 and 6, though failing to respond to the

diuretics by an increase in the formation of urine, had a flow of urine of the same rate that existed before the use of a diuretic solution.

At the second period of observation, $1\frac{1}{2}$ hours after the commencement of the anesthesia, the two animals of Experiments 3 and 5, which had shown a diuretic effect from pituitrin showed but a slight reduction in their alkali reserve and remained diuretic. In these animals the alkali reserve had only been reduced from 8.3 to 8.2. The two animals of Experiments 4 and 6, which had shown no diuretic effect from either caffeine or pituitrin, showed a reduction in the alkali reserve of from 8.25 to 8 and from 8.3 to 8.1. These variations in the rapidity with which the alkali reserve is used up by the anesthetized animals would indicate that the animals which continued to form urine, but which showed no increase in the output of urine from the diuretic solutions were forming during the anesthesia a larger amount of acid than were the animals which not only remained diuretic, but retained their responsiveness to diuretic substances. The tolerance for an alkali as indicated by the rapidity with which the alkali reserve is depleted is greater in the animals non-responsive to diuretics than in the animals in which the use of the diuretic solutions resulted in an increased formation of urine.

At the final period of observation, 2 hours after the commencement of the anesthesia, two of the animals which had received the carbonate protection prior to the anesthetic remained diuretic (Experiments 3 and 5). These animals had a reserve alkali reading of 8 and 7.95. The tension of alveolar air carbon dioxide for the first animal was 32 mm. and for the second animal 34 mm. The animals of Experiments 4 and 6, which also received the carbonate protection, at this stage of the experiment showed a rapid depletion in their alkali reserve and an associated decrease in the tension of alveolar air carbon dioxide. The animal of Experiment 4 had an alkali reserve of 7.85 and a tension of alveolar air carbon dioxide of 24 mm. The readings for the animal of Experiment 6 were practically the same. The alkali reserve had been reduced to 7.85, and the carbon dioxide tension to 21 mm. Both of these animals had become anuric.

At this stage of the experiments the animals were again given diuretic solutions. The animals of Experiments 3 and 5 were given respectively a solution of theobromine and a 0.9 per cent solution of

TABLE II.

Protection of the Naturally Nephropathic Kidney against the Toxic Effect of an Anesthetic by Sodium Carbonate.

Experiment No.	Na^2CO_3 or NaCl solution.	Anesthetic (Grél hant's).	Urine per min.	pH 1 hr. after anesthetic.	R. pH 1 hr. after anesthetic.	Urine 1 hr. after anesthetic.	Diuretic.	Urine per min.	pH 1½ hrs. after anesthetic.	R. pH 1½ hrs. after anesthetic.	Urine 1½ hrs. after anesthetic.	Diuretic.	Urine per min.	pH 2 hrs. after anesthetic.	R. pH 2 hrs. after anesthetic.	Carbon dioxide 2 hrs. after anesthetic.	Urine per min.	Fat in renal epithelium.
		per cent	*gtt.*			*gtt.*		*gtt.*			*gtt.*		*gtt.*			*mm.*	*gtt.*	
1 (control).	NaCl sol. 25 cc. per kg.	60	0	7.35	7.9	0	Theobromine 1%.	0	7.35	7.85	0	Urea sol. 0.9%.	0	7.2	7.7	19	0	V.L.*
2 (control).	NaCl sol. 25 cc. per kg.	60	0	7.4	7.9	0	Pituitrin 0.5 cc.	0	7.25	7.85	0	Theobromine 1%.	0	7.2	7.6	15	0	"
3	Na_2CO_3 sol. 25 cc. per kg.	60	2	7.65	8.3	2	" 0.5 "	3	7.4	8.2	1	Theobromine 1%.	1	7.35	8.0	32	2	Tr.
4	Na_2CO_3 sol. 25 cc. per kg.	60	3	7.5	8.25	2	Caffeine 1%.	2	7.3	8.0	0–1	Glucose sol. 20%.	0	7.3	7.85	24	0	L.
5	Na_2CO_3 sol. 25 cc. per kg.	60	1	7.5	8.3	1	Pituitrin 0.5 cc.	2	7.4	8.2	1	Urea sol. 0.9%.	1	7.3	7.95	34	2	Tr.
6	Na_2CO_3 sol. 25 cc. per kg.	60	1	7.65	8.3	1	" 0.5 "	1	7.4	8.1	1	" " 0.9%.	0	7.35	7.85	21	0	L.

* V. L., indicates very large amount; L., large amount.

urea. To these diuretic substances the animals were responsive. In both experiments the output of urine was increased from one to two drops per minute. The animals of Experiments 4 and 6, which had become anuric, were given either a 20 per cent solution of glucose or a 0.9 per cent solution of urea. These solutions were of no diuretic value. The animals remained anuric.

The histological examination of the kidneys of the animals which have been successfully protected against the toxic effect of the anesthetic by a solution of sodium carbonate shows the type of chronic glomerular pathology which has been previously described. The epithelium of the convoluted tubules shows only a slight degree of swelling and albuminous degeneration. The epithelium is not vacuolated, and the cells show no advanced degenerative changes indicative of a beginning necrosis. The cell cytoplasm stains uniformly and the nuclei take an intense stain (Figs. 1 and 2).

The kidneys of the animals which have shown an early protection against the anesthetic but which later in the experiments have shown a lack of protection by failing to respond to diuretic solutions and by finally becoming anuric, have like the control animals developed an acute swelling, vacuolation, and necrosis of the convoluted tubule epithelium, and have shown a large amount of fat in the ascending limbs of Henle's loops (Fig. 3).

DISCUSSION.

An analysis of the results obtained in the eighteen naturally nephropathic animals which received a solution of sodium carbonate in an attempt to protect them against the toxic effect of an anesthetic shows that based upon the efficiency of the protection the animals may be divided into two groups. All the animals for an hour after the development of a state of anesthesia remained diuretic. This is in striking contrast to the control animals that received the solutions of sodium chloride. At this period of the experiments all the latter animals had become anuric. After this first hour period, however, the animals which received the carbonate solution while remaining diuretic show a variation in the degree of protection conferred by the carbonate in that twelve of the eighteen animals which constitute one

group were responsive to diuretic substances and showed an increased formation of urine when the solutions were employed, while four of the total number of animals representing the second group gave no diuretic response to the same diuretic solutions. It is important to note that at this period of the experiments the reserve alkali of these animals had undergone a depletion, yet the depletion was not below the point of a normal hydroxyl ion content. We cannot, therefore, ascribe this lack of functional response to an acid intoxication in the sense that a sufficiently large amount of hydrogen ions had been liberated during the anesthesia to reduce the alkali reserve below the point of normality. During the remainder of the experiments these animals which early in the anesthesia failed to show an increase in the formation of urine from diuretic substances, show a rapid depletion in their alkali reserve, and become anuric.

The group of animals that received the carbonate solution and were effectively protected against the toxic effect of the anesthetic maintained for a longer period during the anesthesia the increase in the hydroxyl ion content of the blood. At the end of the experiments these animals showed a reserve alkali which was not depleted below 7.95. During the anesthesia and at the termination of the experiments the animals were not only forming urine but they were responsive to diuretic substances.

The present investigation has shown that naturally nephropathic animals may be protected in varying degrees against the toxic effect of an anesthetic by the use of an alkaline solution and that a failure to protect such a kidney during an anesthesia is associated with a rapid depletion of the blood of its alkali reserve and the development of an acid intoxication. This change in the acid-base equilibrium of the blood in these animals has in turn been associated with an acute swelling and necrosis, particularly of the convoluted tubule epithelium, and the development of an anuria. From this observation there is no evidence which would justify the conclusion that the increase in hydrogen ions acting as such upon the epithelial element of the kidney is the cause for the acute swelling and necrosis of the epithelium. The actual way in which an increase of hydrogen ions leads to an injury of the epithelium and the mode of action of an alkaline solution in deferring or preventing this injury remains a problem for future solution.

CONCLUSIONS.

1. A 0.9 per cent solution of sodium chloride when given intravenously to anesthetized naturally nephropathic animals is not effective in preventing the development of an acid intoxication and the associated kidney injury.

2. A solution of sodium carbonate equimolecular with a 0.9 per cent solution of sodium chloride when given intravenously to anesthetized naturally nephropathic animals confers a variable degree of protection to the kidney.

3. The degree of protection conferred by the alkaline solution is associated with the ability of the solution to maintain a normal acid-base equilibrium of the blood of the anesthetized animal.

EXPLANATION OF PLATES.

PLATE 51.

FIG. 1. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of Experiment 3, Table II. It shows at *a*, a glomerulus greatly increased in size from the formation of connective tissue. The capsule is but slightly thickened. The chronic glomerular pathology was mainly of the intracapillary type of change. At *b* are shown convoluted tubules the epithelium of which stains normally and shows no swelling. At *c* are shown tubules the epithelium of which is slightly swollen and stains imperfectly. The kidneys of this animal were successfully protected against the anesthetic by a solution of sodium carbonate. The kidneys were responsive to both pituitrin and theobromine.

PLATE 52.

FIG. 2. Camera lucida drawing, Zeiss oc. 3, obj. 6. The figure is from the kidney of Experiment 5, Table II. It shows at *a* the glomerulus which is increased in size from the formation of connective tissue. Many of the capillary loops have been obliterated. At *b* is shown the thickened capsule. At *c* is shown a dense mass of periglomerular connective tissue. The epithelium of the convoluted tubules at *d* shows but slight swelling. The cytoplasm and nuclei of the cells stain well. At *e* are shown two convoluted tubules in which the epithelium is increased in size without becoming vacuolated. The kidneys of this animal were successfully protected against the anesthetic by sodium carbonate. The animal remained diuretic to pituitrin and a solution of urea.

PLATE 53.

FIG. 3. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of Experiment 6, Table II. The figure shows at *a* the glomerulus which is greatly increased in size from the formation of connective tissue. Many of the capillary loops have become obliterated. The tuft of capillaries is adherent to the greatly thickened capsule *b*. The convoluted tubules at *c* show an acute swelling, vacuolar degeneration, and a beginning necrosis. The kidneys of this animal were imperfectly protected against the anesthetic by a solution of sodium carbonate. The alkali reserve of the blood was rapidly depleted to a reading of 7.85, and the animal became anuric and failed to show a diuretic effect from pituitrin and a solution of urea.

FIG. 1.

FIG. 2.

FIG. 3.

[Reprinted from The Journal of Experimental Medicine, November 1, 1918,
Vol. xxviii, No. 5, pp. 547–550.]

EXPERIMENTAL CHEMICAL PNEUMONIA.

By MARTHA WOLLSTEIN, M.D., and S. J. MELTZER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 55 and 56.

(Received for publication, July 28, 1918.)

The employment of solutions of chloramine-T to remove meningococci[1] and other pathogenic microorganisms from the nasopharynx suggested to us its trial in the treatment of experimental pneumonia, Lobar pneumonia was produced in dogs by means of intrabronchial insufflation of a broth culture of a highly virulent Pneumococcus Type I, invariably leading to a fatal termination. The dose was 2.5 cc. of an 18 to 24 hour culture per kilo of body weight. The autopsy on untreated control animals, which usually succumbed within 48 hours, showed a lobar consolidation in the stage of red hepatization of one lobe of the lung, commonly the lower right lobe. The lower left lobe was less frequently affected, and the subcardiac lobe was only occasionally involved, together with one other. The lesions were microscopically typical of experimental lobar pneumonia in the dog.[2] The pneumococcus insufflated was recovered in pure culture from the solid lung area and the blood of the heart.

The next step was to apply the chloramine-T solution to the inoculated lung. The plan was to introduce 5 cc. per kilo of body weight of a 1:10,000 solution intrabronchially 2 hours after the culture was given, and if the animal survived until the next day or longer, to repeat the injection.

Six animals with the experimentally induced pneumonia were given the chloramine-T solution; they were controlled by three untreated dogs. The results can be briefly stated. One animal

[1] Gordon, M. H., and Flack, M., *Brit. Med. J.*, 1916, ii, 673. Dakin, H. D., *ibid.*, 1917, i, 833.

[2] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

received four, another three, and two others two treatments, while two died after the first dose.

Instead of a therapeutic action, the effects of the treatment were rather to intensify the pathological process. The consolidated areas involved more than one lobe and were attended by much greater edema and hemorrhage than were present in the controls. The animal which survived longest (2 days) and received four injections of the chemical, showed pus in both pleural cavities. Moreover, death resulted, as a rule, more quickly in the treated than in the control animals.

This action of the chloramine-T on the infected lungs led us to a study of the effects of this chemical on lungs of normal dogs. The quantity insufflated was 5 cc. per kilo of a 1:10,000 solution of chloramine-T in normal saline solution. The animals were killed with chloroform at intervals varying from 20 hours to 30 days. One dog of the series died on the 2nd day.

After 24 hours the injected lungs showed areas of consolidation. Usually one lobe was affected, but sometimes two or three lobes were involved (Fig. 1). The lesion proved to be bronchopneumonia, attended by marked congestion and edema. The microscope showed that the alveoli contained red corpuscles, epithelial cells, and polymorphonuclear leukocytes. Very little fibrin was present. The capillaries and larger blood vessels were congested and an excess of leukocytes invaded the alveolar walls.

The 48 hour lesion was practically identical with the preceding (Fig. 2). The 72 hour lesions showed less congestion and beginning resolution. The 4 day specimens were more advanced in resolution, while the 7 day specimens were no longer solid and exhibited only decolorized areas to indicate the seat of the previous consolidation. Two dogs were killed with chloroform on the 30th day. They presented a small wedge of organizing (unresolved) pneumonia in the lower right lobe, possibly a residue from the injections. The chloramine-T pneumonia did not cause fever or any appearance of illness in the animals.

A series of experiments was also made with Dakin's hypochlorite of sodium solution (0.48 per cent) in insufflations of 5 cc. per kilo of body weight. The lung lesions produced by insufflation of this

solution were similar to those produced by the chloramine-T solution. They ran about the same course and resolution was apparent on the 3rd day.

SUMMARY AND CONCLUSIONS.

It is obvious from the experiments reported that concentrations of chloramine-T solutions, even more dilute than those which are well borne by the nasopharyngeal mucosa, are injurious to the pulmonary tissue when introduced directly into the bronchi in large volume. The pulmonary lesions produced are of the nature of an extensive bronchopneumonia which progresses during the first 2 days after injection of the chemical and then regresses, to disappear, as a rule, by the 7th day. Similar pulmonary lesions were produced by intrabronchial insufflation of Dakin's solution of hypochlorite of sodium.

These studies taken in conjunction with the earlier ones of Kline and Meltzer[3] in which aleuronat, starch, egg yolk, and lecithin were insufflated into the lungs, show that pulmonary inflammation may be induced by various chemical substances—with the following differences. Aleuronat and starch set up consolidations containing much fibrin, as is the case with virulent pneumococci; egg yolk and lecithin gave lesions with little fibrin, as is the case with avirulent pneumococci. Both series of substances produced lobar pneumonia, while the pulmonary lesions induced by chloramine-T and sodium hypochlorite were of the nature of bronchopneumonia.

The consolidations of the lung produced by chemical substances differ from infectious pulmonary inflammations only in their sterility. These experimental results strongly suggest the view that the anatomical findings in pneumonia represent a part of a mechanism of defense and repair which the animal body creates in its struggle against infection and intoxication.

[3] Kline, B. S., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1915–16, xiii, 29.

EXPLANATION OF PLATES.

PLATE 55.

FIG. 1. Lungs 24 hours after intrabronchial injection of 1 : 10,000 chloramine-T solution. Dose 5 cc. per kilo of body weight. Right lower lobe swollen, heavy, and solid. Areas of congestion in right upper, and middle lobes. Left lung well aerated.

PLATE 56.

FIG. 2. Section of consolidated lung on the 2nd day after the intrabronchial injection of 1 : 10,000 chloramine-T solution. Dose 5 cc. per kilo of body weight. The intraalveolar exudate consists of epithelial cells, polynuclear leukocytes, and erythrocytes. The alveolar walls are infiltrated with polynuclear cells.

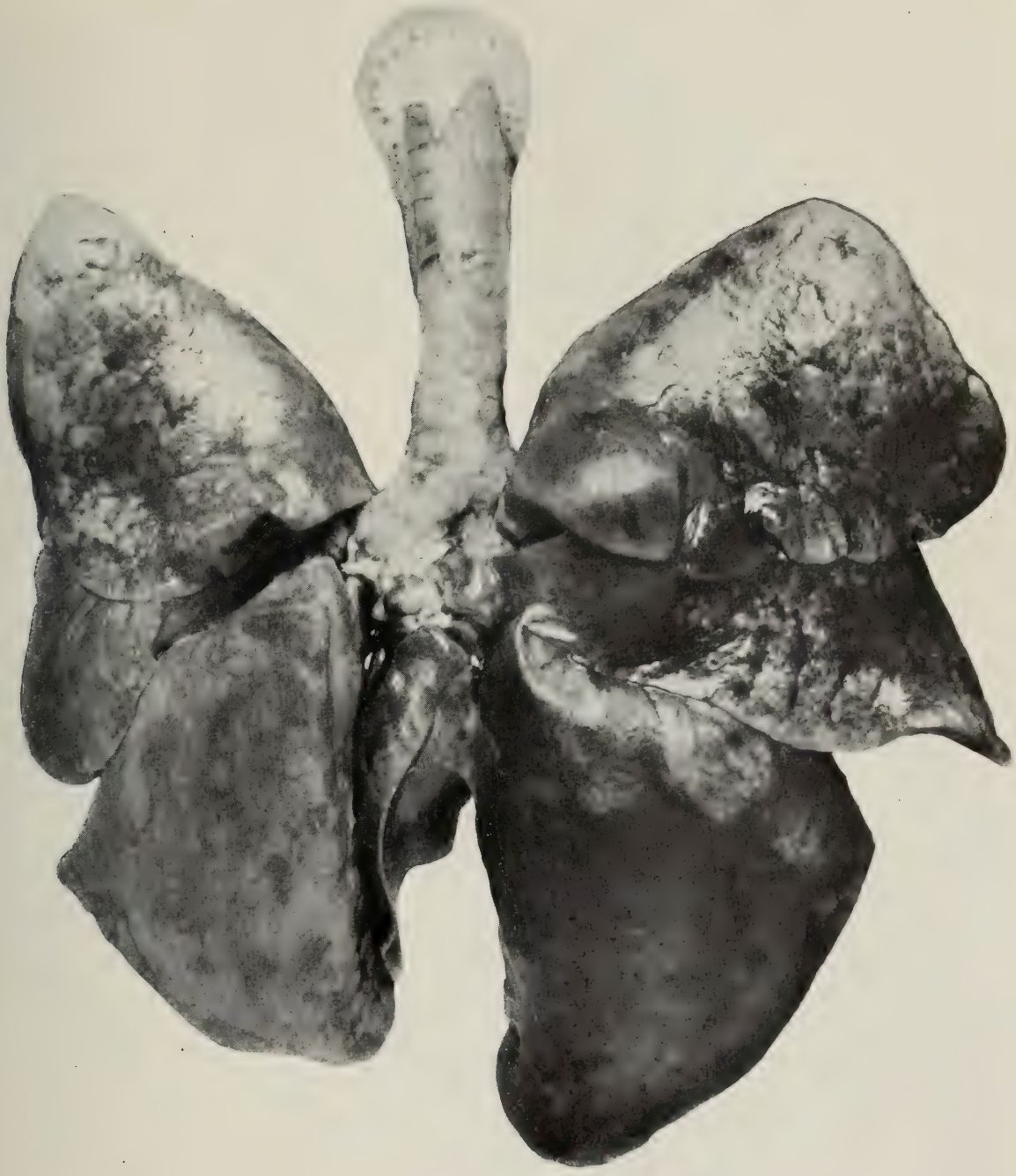


FIG. 1.

(Wollstein and Meltzer: Experimental chemical pneumonia.)

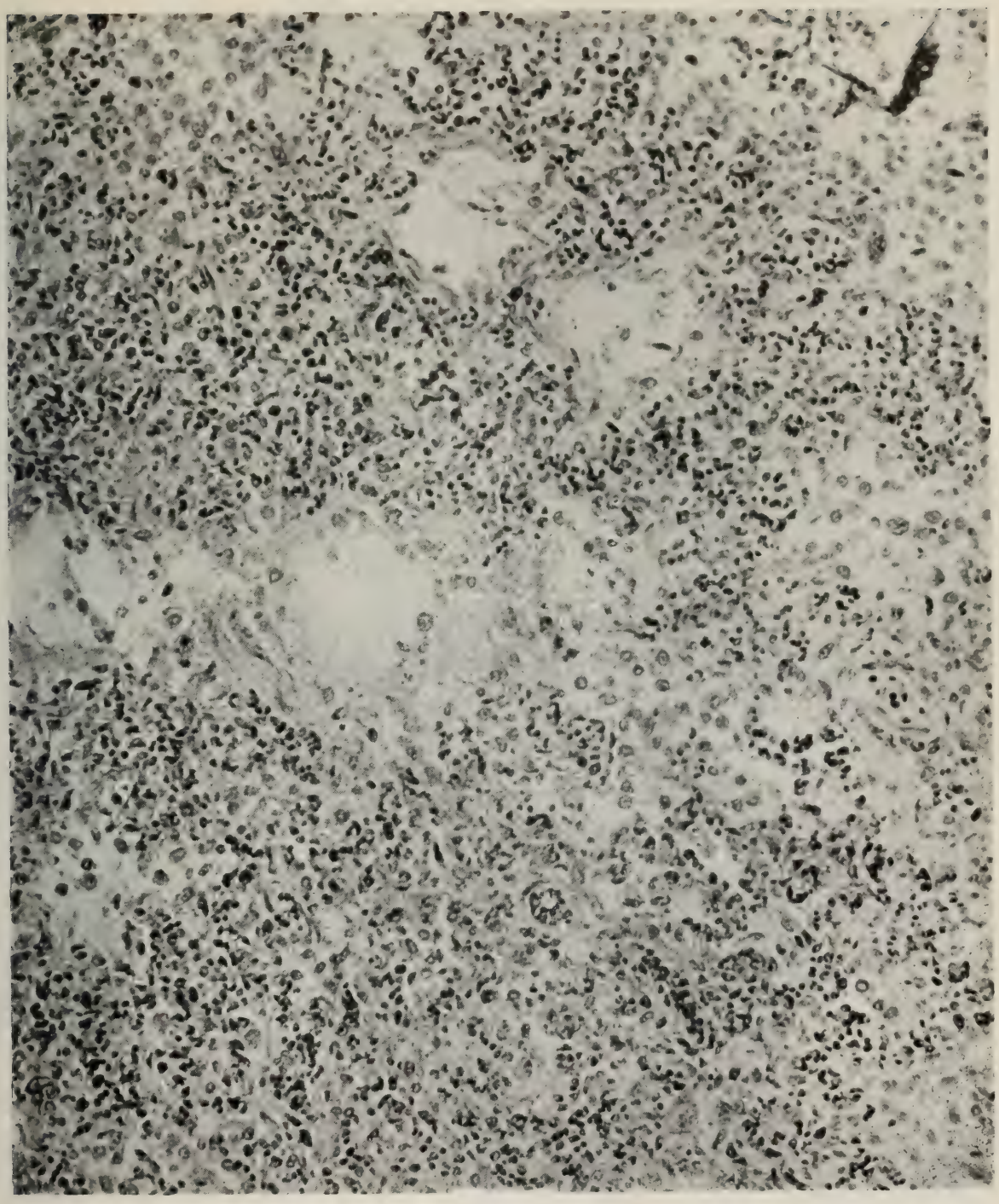


FIG. 2.

(Wollstein and Meltzer: Experimental chemical pneumonia.)

[Reprinted from The Journal of Experimental Medicine, November 1, 1918,
Vol. xxviii, No. 5, pp. 551–558.]

EFFECT OF INTRABRONCHIAL INSUFFLATION OF SOLUTIONS OF SOME INORGANIC SALTS.

By MARTHA WOLLSTEIN, M.D., and S. J. MELTZER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 28, 1918.)

Lamar and Meltzer[1] stated that bronchial insufflation of an isotonic solution of sodium chloride produces no lesion in the lungs. Observations were also made in the course of various experiments carried on by us, which justify the statement that neither hypotonic solutions of sodium chloride nor distilled water causes any pulmonary lesions. In drawing conclusions from these studies the fact should not be forgotten that the posterior part of the lower lobes even of normal dogs frequently shows at autopsy, as previously stated by us,[2] a slight hyperemia when the animals are killed with chloroform.

However, the experience we gathered from the insufflation of sodium hypochlorite caused us to investigate the action of some other inorganic salts in hypotonic and hypertonic solutions. The results are briefly recorded here.

Mercuric Chloride.

Bichloride of mercury was employed in high dilution—1 : 10,000. 5 cc. per kilo of body weight of this solution were insufflated into the bronchi of dogs in the usual manner. At autopsy there was always an unmistakable pulmonary lesion. When the animal was killed after 24 hours the affected lobe of the lung was heavy, dark red in color, and edematous. Microscopically the lesions proved to be largely hemorrhagic in character, and thrombi were present in some of the blood vessels. After 48 hours the hemorrhage and edema were more pronounced, but by the 3rd day the lesion had begun to regress. No bacteria grew in cultures made from the lungs. Obviously the lesion

[1] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

[2] Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1913, xvii, 424.

produced by the injection of mercuric chloride directly into the bronchi is not an inflammation; it is merely a hemorrhage which may be so great as to rupture the alveolar walls. This condition is associated with or perhaps caused by thrombosis of the blood vessels.

2 Per Cent Sodium Chloride Solution.

In a series of eight dogs the effect of intrabronchial insufflation of sodium chloride in hypertonic solution (2 per cent equal to about 0.34 M) was tested. 5 cc. of the solution per kilo of body weight were insufflated. Part of the animals were killed 24 hours and others 48 hours after the insufflation. Three of the dogs showed a small area of bronchopneumonia in one lower lobe, while in four others the lungs were practically normal. The eighth animal died soon after the insufflation.

Experiment 1.—December 24, 1917. Black dog, female; weight 5.550 kilos. Received an intrabronchial injection of 28 cc. of sodium chloride, 2 per cent. Killed with chloroform 24 hours later.

Autopsy.—In the right lower lobe there was a wedge-shaped area in the inferior posterior angle measuring 3 by 4 cm. in diameter; it was red, heavy, and swollen.

On section the appearance of the cut surface was that of red bronchopneumonia. Edema and congestion were practically absent from the rest of the lungs.

Microscopic examination proved the lesion to be one of bronchopneumonia, with hemorrhage into some alveoli, and only a small amount of edema.

Experiment 2.—December 24, 1917. Male dog; weight 5.800 kilos. Received intrabronchial insufflation of 30 cc. of sodium chloride, 2 per cent. December 26. Killed with chloroform.

Autopsy.—The right lower lobe contained a wedge in its posterior inferior angle which was not raised, but was dark red in color, and solid.

The cut surface had the appearance of a bronchopneumonia. There was some congestion, but no redness in the other lobes of both lungs.

Microscopic examination showed early bronchopneumonia; the alveoli contained peeled epithelium, red blood cells, granular material, and leukocytes. Other places were more solid, with more leukocytes in the alveoli and infiltrating the walls. The lining cells of the bronchi were normal. There was some periarterial edema.

Experiment 3.—January 3, 1918. Female dog; weight 7.550 kilos. Received intrabronchial insufflation of 38 cc. of sodium chloride, 2 per cent. Killed with chloroform after 24 hours.

Autopsy.—In the posterior inferior angle of the left lower lobe there was a wedge of dark red bronchopneumonia; this area was neither swollen nor heavy; it was

very moist, and the entire lobe was edematous. The right lower lobe contained an area of congestion but no consolidation. Other lobes were normal; there was no pleurisy.

Experiment 4.—January 3, 1918. Male dog; weight 6.300 kilos. Intrabronchial injection of 38 cc. of sodium chloride, 2 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs were pink and well aerated.

Experiment 5.—January 8, 1918. Male dog; weight 6 kilos. Intrabronchial injection of 30 cc. of sodium chloride, 2 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs showed no consolidation at any point and no edema; very mild congestion.

Experiment 6.—June 17, 1918. Male dog; weight 8.300 kilos. Intrabronchial injection of 40 cc. of sodium chloride, 2 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs showed moderate congestion of the right lower lobe. All the other lobes were normal.

Experiment 7.—June 17, 1918. Male dog; weight 9.750 kilos. Intrabronchial injection of 50 cc. of sodium chloride, 2 per cent. Killed with chloroform after 24 hours.

Autopsy.—There was a small amount of congestion in both lungs and in the trachea. Otherwise there were no lesions. Edema was not present.

Experiment 8.—January 18, 1918. Male dog; weight 12.050 kilos. Intrabronchial injection of 60 cc. of sodium chloride, 2 per cent. Found dead after 40 minutes.

Autopsy.—Both lower lobes and the subcardiac lobe were full, heavy, dark red, but translucent. Frothy fluid ran from the trachea.

On section the lungs showed very marked edema, and moderate congestion. There was no bronchitis and no pneumonia. This dog apparently was simply drowned by the intrabronchial injection.

The cultures from the lungs of all the dogs were sterile. Of seven dogs which received intrabronchially the same quantity of a hypertonic solution of sodium chloride, four showed practically normal lungs. The occasional slight congestion observed in these animals may have been due to the use of chloroform in killing them. Three dogs, however, showed lesions. Although the inflammatory area was comparatively small, its bronchopneumonic nature was unmistakable. Attention ought perhaps be called to the fact that these three instances occurred in December and January. It is possible that at this time of the year the lungs are more readily affected by a slight irritant.

As a whole our experiments justify the statement that a hypertonic solution of sodium chloride, administered by intrabronchial injection, causes a comparatively unimportant lesion of the lungs, or none at all.

11 Per Cent Sodium Sulfate Solution.

Five dogs received 5 cc. per kilo of body weight of an 11 per cent solution of sodium sulfate intrabronchially.

Experiment 9.—December 24, 1917. Male dog; weight 6.150 kilos. Received 31 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs were well aerated; there were a few small, dark, congested lobules and practically no edema.

Experiment 10.—December 24, 1917. Female dog; weight 7.300 kilos. Received intrabronchial injection of 38 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs were pink and well aerated. A few small congested areas were present but there were no consolidation and no edema.

Experiment 11.—January 3, 1918. Female dog; weight 6.700 kilos. Intrabronchial injection of 40 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 48 hours.

Autopsy.—Only the subcardiac lobe was solid with red bronchopneumonia, and some edema. All the other lobes were well aerated.

Experiment 12.—January 3, 1918. Female dog; weight 7.450 kilos. Injected intrabronchially with 48 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 24 hours.

Autopsy.—The right lower lobe contained a wedge-shaped area of bronchopneumonia, rather gray and very moist; there was much edema. No lesion in the other lobes.

Experiment 13.—January 8, 1918. Male dog; weight 8.600 kilos. Intrabronchial injection of 45 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 24 hours.

Autopsy.—The subcardiac lobe was translucent, dark red in color.

On section, it showed bronchopneumonia with plugs of mucus in the bronchi. In the other lobes there was a moderate amount of congestion and edema but no consolidation. A general bronchitis was present. The animal undoubtedly suffered from distemper.

In the first two experiments of this series the intrabronchial injection of a distinctly hypertonic solution of sodium sulfate caused no lesion whatsoever. The last experiment was an instance of distemper. It is not impossible that the dogs in Experiments 11 and 12 were not

normal when the experiment was begun. At any rate, the experiments seem to justify the general statement that an intrabronchial injection of a hypertonic solution of sodium sulfate, like a similar solution of sodium chloride, causes either no pulmonary lesion at all or only an insignificant one.

Magnesium Salts.

A few experiments were made with intrabronchial insufflation of magnesium salts. It should be borne in mind that the intrabronchial use of these salts is fraught with danger. In the first place, it may cause deep anesthesia and paralysis of respiration. The latter could, however, be obviated by intravenous or intramuscular injection of calcium chloride. More serious is the paralytic effect which the magnesium salt may exert upon the heart. Coming directly in contact with the capillaries of the lung, the solution of magnesium salts, without previously being diluted by the blood of the right ventricle, enters the left ventricle and its coronary artery and may thus cause a fatal effect upon the activity of the left ventricle. Two experiments were made with magnesium sulfate and four with magnesium chloride.

Experiment 14. Magnesium Sulfate.—September 12, 1917. 5 cc. per kilo of body weight of hydrated magnesium sulfate, containing seven molecules each of water of crystallization, were introduced intrabronchially. The animal was etherized by intratracheal insufflation and at 10.12 a.m., a cannula was introduced into the jugular vein, to be ready for an injection of calcium chloride. 12 m. 50 cc. of a 6 per cent solution of magnesium sulfate were introduced intrabronchially. 12.27 p.m. The animal was awake but not struggling; placed on the floor. 12.54 p.m. Still recumbent but the head was up. The cannula was removed and the wound stitched. 5 p.m. Still lying down; coughing; temperature 40.2°C. September 13, 9 a.m. No coughing; quiet; temperature 39.3°C. September 14, 9 a.m. Temperature 39.5°C. Condition improved. 11.30 a.m. Killed with chloroform (48 hours after intrabronchial injection of the salt).

Autopsy.—The posterior part of the right lower lobe was half solid, red, swollen, moderately congested, not very heavy, not friable.

The cut surface showed lobular pneumonia, which was confirmed by microscopic examination.

Experiment 15. Magnesium Sulfate.—January 8, 1918. Female dog; weight 10.650 kilos. Injected intrabronchially with 56 cc. of magnesium sulfate, 8.38 per cent, followed by an intramuscular injection in the right thigh of 10 cc. of calcium chloride, 2.5 per cent. January 9, 9 a.m. Animal quiet, lying down;

temperature 39.2°C. January 10, 9 a.m. Temperature 39.5°C.; quiet. 10.30 a.m. Killed with chloroform (about 24 hours after injection of the solution of magnesium sulfate).

Autopsy.—Right lower lobe heavy, swollen, edematous, and congested; not very solid.

The cut surface was mottled with bronchopneumonic areas, especially in the upper part of the posterior border. A moderate amount of congestion was present in the right upper and middle lobes.

Experiment 16. Magnesium Chloride.—January 8, 1918. Male dog; weight 6.850 kilos. 11.44 a.m. Injected intrabronchially with 35 cc. of magnesium chloride, 6.92 per cent (about 5 cc. per kilo), followed by an injection into the right thigh of 10 cc. of calcium chloride, 2.5 per cent. 4 p.m. Temperature 40.1°C. January 9, 9 a.m. Temperature 38.5°C. Animal lively. 10.30 a.m. Killed with chloroform (about 24 hours after injection of magnesium chloride solution).

Autopsy.—The apex of the right upper lobe was solid and swollen; on section very dark areas of hemorrhage alternated with pale red areas of bronchopneumonia. The lower lobe was less solid than the upper; on section it was mottled with areas of bronchopneumonia; there were marked edema and moderate congestion.

Experiment 17. Magnesium Chloride.—June 24, 1918. Female dog; weight 8.150 kilos. Injected intrabronchially with 33 cc. of magnesium chloride, 6 per cent (about 4 cc. per kilo). No rise of temperature followed. The dog was killed with chloroform after 24 hours.

Autopsy.—The right lower lobe contained a firm, dark blue area in the posterior inferior angle, 3 to 4 cm. in diameter. On section, this showed bronchopneumonia with marked edema; the area was red in color and congested moderately. A small amount of frothy fluid in the trachea and the main bronchi.

Experiment 18. Magnesium Chloride.—June 24, 1918. Female dog; weight 14.050 kilos. Given an intrabronchial injection of 56 cc. of magnesium chloride solution, 6 per cent (about 4 cc. per kilo). No rise in temperature followed. June 26, 11 a.m. Animal normal; killed with chloroform (48 hours after injection of magnesium chloride).

Autopsy.—There was a small superficial area of congestion in the left lower lobe about the middle of the posterior border. All the other lobes were well aerated, slightly congested, and not edematous. No frothy fluid in the trachea.

Cultures made from the heart and lungs of the animals intrabronchially injected with magnesium salts showed no growth.

Two more experiments with insufflation of magnesium chloride were made, but they do not belong to this series. They are recorded merely to show the danger of an intrabronchial insufflation of a concentrated solution of a magnesium salt. Through an error a 6 per cent solution of magnesium chloride was prepared from a dehydrated

salt. Without knowing this fact the solution was used on two dogs, giving 4 cc. per kilo. The first dog died a few minutes after the insufflation. The history of the second dog follows:

Experiment 19.—Male dog; weight 12.950 kilos. June 17, 1918, 2 p.m. An intrabronchial injection of 50 cc. of magnesium chloride, about 14 per cent (about 4 cc. per kilo), was given. 2.30 p.m. There was no spontaneous respiration and intratracheal insufflation was started. Froth was emitted from the nose and mouth. Given 30 cc. of calcium chloride, 2.5 per cent, into the muscles of the right thigh. 2.55 p.m. Spontaneous respirations appeared and became regular; lid reflex fair. The insufflation was discontinued, but the tube was left in the trachea for 30 minutes, then removed. 5 p.m. Temperature 37.3°C.; lying down, sick. June 18, 9 a.m. Temperature 39.9°C. 5.45 p.m. Found dead.

Autopsy.—Performed next day. No consolidation in the lungs. The right lower lobe was slightly decolorized and full in the posterior portion. On section, it was congested. Frothy fluid was present in the bronchi. The left lung and the trachea contained no fluid. The 30 cc. of calcium given intrabronchially may have prevented for a time the fatal effect of the magnesium.

From the experiments given above it would seem that magnesium salts are more liable to produce (moderate) pulmonary lesions than sodium salts and that magnesium sulfate may be more effective than magnesium chloride. However, we must bear in mind that the number of experiments in these series is too small to draw definite conclusions from them. For the same reason we shall not attempt to discuss whether this effect of magnesium sulfate upon the lung could be connected with the observation of Gates[3] on the production of hyaline casts by intravenous injections of magnesium sulfate.

SUMMARY.

Intrabronchial injections of isotonic as well as of hypotonic solutions of sodium chloride or even of distilled water cause no pulmonary lesions.

Intrabronchial injections of mercuric chloride even in a dilution of 1 · 10,000 cause a marked pulmonary lesion. The lesion is not of an inflammatory character; it consists of congestion, formation of thrombi, and hemorrhage.

[3] Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1913–14, xi, 102. See also Gates, F. L., and Meltzer, S. J., *ibid.*, 167.

Intrabronchial injections of hypertonic solutions of sodium chloride as well as of sodium sulfate cause, in most instances, no lesions whatsoever. In a smaller number of cases in which moderate lesions were present they may have been due either to a previous infection (distemper) or to some predisposing cause (winter months).

Intrabronchial injection of magnesium salts apparently tends to cause moderate pulmonary lesions (bronchopneumonia). This seems especially true of magnesium sulfate..

[Reprinted from The Journal of Experimental Medicine, November 1, 1918,
Vol. xxviii, No. 5, pp. 559–560.]

THE SPIROCHETAL FLORA OF THE NORMAL FEMALE GENITALIA.

By HIDEYO NOGUCHI, M.D., and DAVID J. KALISKI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 57.

(Received for publication, June 27, 1918.)

The present paper is a sequel to a recently published[1] study of the spirochetal flora of the male genitalia. The subject has been taken up, not because there was any reason for supposing the spirochetal flora of the female genitalia to be different from that of the male, but because of the relative infrequency of any special reference to the former in literature, and in order to obtain more accurate knowledge concerning it. Except for the occurrence of the well known *Spironema refringens* (Schaudinn and Hoffmann[2]) in both male and female genitalia, practically nothing is known about the spiral organisms in the normal female genitalia.

On anatomic and physiologic grounds the female genitalia undoubtedly afford more favorable conditions for the existence of these saprophytic spirochetes, and the results of examinations of the smegma, films, and washings of the genital mucous membranes of normal adult females conclusively demonstrate that the number of spirochetes of the female is much greater than that of the adult male, although the varieties present appear to be identical in both. As in the male smegma *Treponema calligyrum* is encountered here also as the predominating variety in the majority of specimens, while *Treponema minutum* is present almost constantly but in fewer numbers. On the other hand, the coarse *Spironema refringens* has been less frequently met with here than in the male smegma. The finding may have been accidental but is recorded here for future reference.

[1] Noguchi, H., The spirochetal flora of the normal male genitalia, *J. Exp. Med.*, 1918, xxvii, 667.

[2] Schaudinn, F., and Hoffmann, E., *Arb. k. Gsndhtsamte.*, 1905, xxii, 527.

The morphologic and cultural characteristics of these three types were described in a previous publication.[1] In Figs. 1 to 8 *calligyrum*, *minutum*, and *refringens* are easily distinguished. The organisms stained by Fontana's method (Figs. 1 to 3) appear more distinctly than those stained by the mordant gentian violet method (Figs. 4 to 8), but in either case there is no difficulty in recognizing the types. Here, as with the male smegma, the use of the dark-field microscope is important in order to avoid confusion of the organisms belonging to the *Leptospira* group with those normally present.

Examination of the spirochetal flora of female children up to the age of 2 years showed that the varieties present are similar to those found in the adult, differing from the latter only in being less numerous.

CONCLUSION.

The spirochetal flora of normal female genitalia is similar to that of the male and consists of *Treponema calligyrum*, *Treponema minutum*, and *Spironema refringens*. The types are present in varying proportions, but the *calligyrum* usually predominates, and the *refringens* is the least frequent. The female genitalia are much richer generally in the number of spirochetes than the male.

EXPLANATION OF PLATE 57.

Magnification, × 1,000.

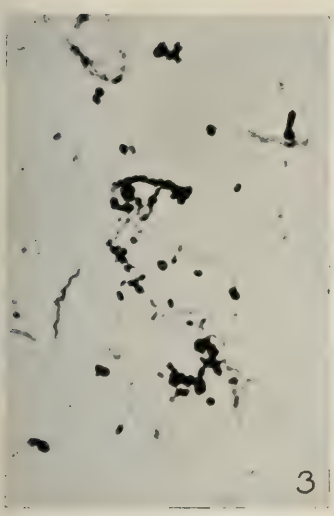
FIGS. 1 to 3. Films from female smegmas, showing *Treponema calligyrum* and *Treponema minutum*.

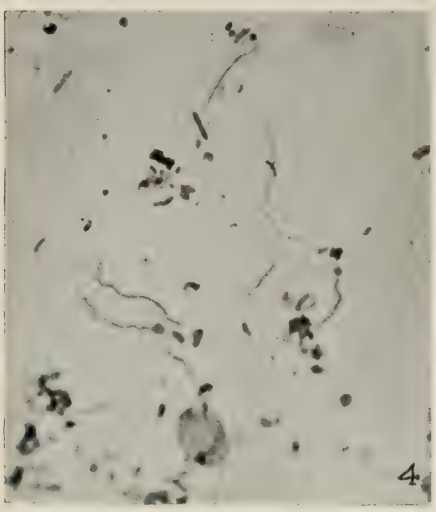
FIG. 4. Several specimens of *Treponema calligyrum* and a few of *Spironema refringens*.

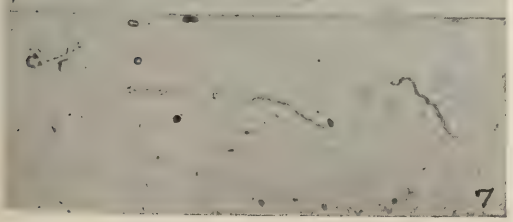
FIG. 5. Several specimens of *Treponema calligyrum* and one of *Treponema minutum*.

FIG. 6. The three types.

FIGS. 7 and 8. *Treponema calligyrum* and *Treponema minutum*.







(Noguchi and Kaliski: Spirochetal flora of normal female genitalia.)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, November 1, 1918,
Vol. xxviii, No. 5, pp. 561–570.]

A COMPARATIVE STUDY OF EXPERIMENTAL PROPHYLACTIC INOCULATION AGAINST LEPTOSPIRA ICTEROHÆMORRHAGIÆ.

BY HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, August 16, 1918.)

Ido, Hoki, Ito, and Wani[1] first demonstrated that when guinea pigs are treated with three successive injections of killed cultures of *Leptospira icterohæmorrhagiæ* at 5 day intervals they become refractory to a subsequent infection, undertaken 10 days later, with a virulent culture or with liver emulsions of infected guinea pigs. After about 17 to 19 days the serum of these animals contained a small amount of immune bodies when tested by the method of Pfeiffer. From this experiment the conclusion was drawn that the degree of immunity induced in these animals is comparable with that which develops in the serum of patients on the 8th day of disease, in the stage when the infective organisms have already disappeared from the peripheral blood, as shown by negative results of animal inoculations with the blood. The immunity is incomplete, but sufficiently strong to protect the animals from the infection. These writers found also that the serum of six persons inoculated with three successive doses of highly concentrated vaccine (carbolized emulsion of the pure culture) for the purpose of prophylaxis contained a demonstrable amount of immune bodies when tested by Pfeiffer's method 10 days after the last inoculation, but when given to unvaccinated guinea pigs the serum failed to protect the animals against a simultaneous infection. There was considerable difference, however, between the course of the disease in the animals receiving the serum from vaccinated persons and those receiving normal human serum, the former outliving the controls by as many as 10 days, the number of spirochetes being fewer, and the icterohemorrhagic lesions less marked.

Ito and Matsuzaki[2] recommended for the purpose of protective vaccination the use of the blood gelatin culture of *Leptospira icterohæmorrhagiæ*. The culture was killed by heating it to 60°C. for 30 minutes on 3 successive days. The killed culture was toxic when given intravenously, and one of the samples killed guinea pigs, in a dose of 0.5 cc. or more per 100 gm. of body weight, within a few minutes. A single subcutaneous injection of 0.05 cc. of another preparation of the killed cul-

[1] Ido, Y., Hoki, R., Ito, H., and Wani, H., The prophylaxis of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiv, 471.

[2] Ito, T., and Matsuzaki, H., Prophylactic vaccination, serotherapy, and chemotherapy in Weil's disease (in Japanese), *J. Chiba Med. College*, 1916, Nos. 83 and 84.

ture, the minimum lethal dose of which was 1.5 cc. per 100 gm. of body weight, was found to be sufficient to confer upon the animal a complete immunity against a subsequent infection after 2 weeks, although there was no protection at the end of the 1st week. Ito and Matsuzaki also prepared a pulverized vaccine from a desiccated blood gelatin culture of the *icterohæmorrhagiæ* and found that it not only retained its immunizing quality but possessed the advantage over the liquid vaccine of being nearly ten times more concentrated. For protective vaccination they used a saline emulsion of the dried culture. They calculated the quantity necessary for a man of 50 kilos body weight to be 25 to 30 cc. of the liquid and 2.5 to 3 gm. of the dried material. The serum from the vaccinated animals contained the specific immune bodies demonstrable by Pfeiffer's procedure and also *in vitro* (Sabritschewsky's phenomenon).

From the experiments of Ido, Hoki, and others on the one hand, and those of Ito and Matsuzaki on the other, it is evident that the guinea pig, which is perhaps the animal most susceptible to infection by *Leptospira icterohæmorrhagiæ*, can be rendered actively immune by a single injection or by multiple injections of devitalized cultures. The question arises as to whether the immunity thus induced with one strain will protect the animal equally well against the other strains. The present work was undertaken to determine the relation of one strain to another by means of cross-immunity reactions among the strains isolated in Japan, America, and Europe.

Method.

For the purpose of protective inoculation guinea pigs weighing about 400 gm. were selected. The emulsions of *Leptospira icterohæmorrhagiæ* were prepared from pure cultures of different strains grown on the rabbit serum Ringer medium (semisolid below and liquid above[3]) at 26°C. for from 3 to 6 weeks. The liquid portion of the culture, showing 20 to 30 organisms per field ($\frac{1}{12}$ oil immersion and Leitz ocular 3), was gathered from a number of tubes and, after immobilization of the organisms by means of 0.4 per cent phenol (determined by the dark-field microscope), was centrifuged for 2 hours at the rate of 4,000 revolutions per minute; the deposit was carefully separated from the supernatant fluid and resuspended in one-fifth

[3] Noguchi, H., Further study on the cultural conditions of *Leptospira (Spirochæta) icterohæmorrhagiæ*, *J. Exp. Med.*, 1918, xxvii, 593.

of the original volume. The emulsion thus concentrated contained a great number of the organisms and was preserved under a layer of toluene for 1 week in the refrigerator (6°C.) before use. The emulsion was then tested by culture in order to be certain that there were no live organisms left.

Emulsions were prepared from two American strains isolated from wild rats caught in the vicinity of New York, one European, and one Japanese strain,[4] and the inoculations were made subcutaneously. In the first group of animals 0.5 cc., in the second 0.05 cc., and in the third 0.005 cc. of the emulsion was given three times at intervals of 5 days. Except in a few instances the animals showed no rise in temperature after the inoculation. As a whole they stood the inoculations well, though a number were lost through secondary infection. Some lost weight during the vaccination period.

Tests for active immunity against the homologous and heterologous strains were made at the end of 2, 4, and 8 weeks after the last inoculation. The quantities used for the injection intraperitoneally into the vaccinated and control (unvaccinated) animals were those containing at least several minimum lethal doses of each strain. For example, in the case of American Strain 1, 0.0005 cc. was used, American Strain 2, 0.005 cc., American Strain 3, 0.05 cc., Japanese, 0.00005 cc., and European, 0.005 cc. of 3 week cultures on the rabbit serum Ringer medium at 26°C. Two control animals accompanied each series of experiments, and they always died of the typical infection within 5 to 7 days.

Notwithstanding the care which they received, some of the vaccinated guinea pigs died of secondary infection, or in a few instances of marasmus, before the experiments were completed.

RESULTS.

The results of the tests are briefly summarized in Tables I to IV.

Table I shows that the guinea pigs receiving three successive injections of 0.05 and 0.5 cc. of the concentrated vaccine of American Strain 1 were, with two exceptions, protected against a subsequent

[4] Noguchi, H., *Spirochæta icterohæmorrhagiæ* in American wild rats and its relation to the Japanese and European strains, *J. Exp. Med.*, 1917, xxv, 755.

TABLE I.

Vaccine Made from American Strain 1.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
American Strain 1	Survived.	Survived.	Died in 7 days.	Died in 6 days.	Survived.	—	Survived.	Died in 6 days.	—	Survived.	Survived.	Died in 7 days.
" " 2	"	Died in 8 days.	Died in 9 days.	Died in 6 days.	"	Survived.	Died in 7 days.	Died in 6 days.	Survived.	"	Died in 8 days.	Died in 6 days.
" " 3	"	Survived.	Died in 8 days.	Died in 5 days.	—*	"	Survived.	Died in 6 days.	"	—	Survived.	Died in 7 days.
Japanese strain.	"	"	Died in 8 days.	Died in 6 days.	Survived.	"	Died in 11 days.	Died in 5 days.	"	Survived.	Died in 7 days.	Died in 5 days.
European "	"	Died in 8 days.	Died in 8 days.	Died in 7 days.	"	"	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.

*— indicates that the animal succumbed to secondary infection.

TABLE II.

Vaccine Made from American Strain 2.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
American Strain 2	Survived.	Survived.	Died in 7 days.	Died in 6 days.	Survived.	Survived.	Died in 9 days.	Died in 7 days.	—	Survived.	Died in 9 days.	Died in 6 days.
" " 1	"	"	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	Survived.	Died in 13 days.	—	Died in 6 days.
" " 3	"	"	Died in 9 days.	Died in 7 days.	"	"	Died in 7 days.	Died in 7 days.	"	—	Died in 7 days.	Died in 7 days.
Japanese strain.	"	Died in 10 days.	Died in 7 days.	Died in 5 days.	"	—	Died in 8 days.	Died in 5 days.	"	Died in 10 days.	Died in 6 days.	Died in 5 days.
European "	"	Survived.	Died in 8 days.	Died in 7 days.	"	Survived.	Died in 8 days.	Died in 6 days.	"	Survived.	Died in 8 days.	Died in 6 days.

TABLE III.

Vaccine Made from Japanese Strain.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
Japanese strain.	Survived.	Survived.	Died in 11 days.	Died in 5 days.	Survived.	Survived.	Survived.	Died in 5 days.	Survived.	—	Survived.	Died in 5 days.
American Strain 1	"	"	—	Died in 6 days.	—	"	Died in 9 days.	Died in 6 days.	"	Survived.	Died in 8 days.	Died in 6 days.
" " 2	"	—	Died in 9 days.	Died in 7 days.	Survived.	"	Died in 10 days.	Died in 7 days.	—	Died in 9 days.	Died in 11 days.	Died in 7 days.
" " 3	"	Survived.	Died in 7 days.	Died in 6 days.	"	"	Died in 12 days.	Died in 6 days.	Survived.	Survived.	Died in 13 days.	Died in 6 days.
European strain.	"	Died in 8 days.	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	"	Died in 12 days.	Died in 8 days.	Died in 6 days.

TABLE IV.

Vaccine Made from European Strain.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
European strain.	Survived.	Survived.	Died in 8 days.	Died in 5 days.	Survived.	Survived.	Survived.	Died in 6 days.	Survived.	Survived.	Died in 12 days.	Died in 6 days.
American Strain 1	"	Died in 12 days.	Died in 8 days.	Died in 6 days.	—	"	Died in 8 days.	Died in 6 days.	"	Died in 9 days.	Died in 8 days.	Died in 6 days.
" " 2	"	Survived.	Died in 9 days.	Died in 6 days.	Survived.	"	Died in 13 days.	Died in 6 days.	—	Survived.	Died in 9 days.	Died in 6 days.
" " 3	"	"	Died in 13 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	Survived.	"	—	Died in 7 days.
Japanese strain.	"	Died in 8 days.	Died in 6 days.	Died in 5 days.	"	—	Died in 6 days.	Died in 5 days.	"	Died in 8 days.	Died in 6 days.	Died in 5 days.

infection undertaken 2, 4, and 8 weeks after the last inoculation. One exception occurred in the case of American Strain 2 and one in the case of the European strain, both animals succumbing to the infection 1 to 2 days later than their respective controls. On the other hand, three injections of 0.005 cc. of the same vaccine protected the animals against the same strain as well as against American Strain 3, but failed to produce complete immunity against the other three heterologous strains.

Table II gives the results obtained with the guinea pigs vaccinated with American Strain 2. The protection was complete in the group receiving 0.05 and 0.5 cc., except when tested against the Japanese strain and American Strain 1, in which instances death was delayed but not prevented. There was no resistance against any strain when the dose of the vaccine was 0.005 cc.

In the series reported in Table III, the highest degree of immunity is seen to have been developed against the Japanese strain, which was the one used as the vaccine in this series. It was the only strain which failed to kill guinea pigs that received injections of the smallest amount of the vaccine (0.005 cc.). Protection was weakest against American Strain 2 and the European strain. Three injections of 0.5 cc. in succession, however, conferred complete immunity against all the strains tested.

A similar relation exists between the homologous and heterologous strains of the next series (Table IV), the protection being most marked against the same organism. All the guinea pigs vaccinated with 0.5 cc. of any one strain resisted infection with that and all other strains used in the present study.

While these results do not furnish basis enough to permit division of the various strains into groups or definite types, yet the general indication is that there is a closer affinity among some strains than others. For example, American Strains 1 and 3 seem to be closely allied, American Strain 2 being nearer to the European strain. The Japanese strain stands between the two groups and much nearer that formed by American Strains 1 and 3.

With respect to the duration of the immunity thus established, it was found that at the end of 8 weeks there was not an appreciable diminution of the immunity, although of the animals receiving smaller amounts of the vaccine a slightly larger number survived when tested at the end of 4 than at the end of 2 or 8 weeks.

SUMMARY AND CONCLUSIONS.

Guinea pigs were inoculated with suspensions of *Leptospira icterohæmorrhagiæ* obtained from pure cultures of several different strains, in order to determine whether or not an active immunity against a subsequent infection with virulent organisms would develop in the vaccinated animals. The experiments were so arranged as to make possible a determination of the existence of immunity against homologous strains as well as against the strains not employed as vaccine, and a brief quantitive estimation of the degree and duration of the immunity in relation to the quantities of the vaccines inoculated. Following the general rule of prophylactic inoculations with various pathogenic organisms, the inoculations were repeated subcutaneously on three consecutive occasions at intervals of 5 days. With respect to the amounts of vaccine, the experiments were divided into three groups for each vaccine, one group receiving three doses of 0.5 cc., the second three of 0.05 cc., and the third three of 0.005 cc. Four different strains were employed as vaccines, American Strain 1, American Strain 2, and one each of the Japanese and the European strains.

The determination of the development, degree, and duration of the immunity was made by inoculating intraperitoneally several minimum lethal doses of each of the five following strains: American Strains 1, 2, and 3, the Japanese, and the European strains. The virulence of the different strains varied considerably, the strongest being the Japanese strain, which killed the guinea pig in a dose of 0.00001 cc., and the weakest American Strain 3, the minimum lethal dose of which was as large as 0.01 cc.

The vaccinated guinea pigs were tested for immunity at the end of 2, 4, and 8 weeks after the last inoculation.

The results obtained show that three successive inoculations of 0.5 cc. of the emulsions of killed cultures of *Leptospira icterohæmorrhagiæ* into guinea pigs rendered them completely resistant to a subsequent infection with the virulent cultures of both homologous and heterologous strains. With 0.05 cc. the protection was not so general, the animals succumbing to an experimental infection with some heterologous strains while resisting the homologous and other heterologous strains. The animals which were vaccinated with 0.005 cc. survived

the infection experiments with the homologous strains in the case of American Strain 1 and the Japanese strain, but they were not protected against any other strains. The vaccines of other strains were unable to immunize the guinea pigs so highly even against their homologous strains, when the amount of each inoculation was only 0.005 cc., but 0.05 cc. conferred complete protection against the same strains. It may be concluded, therefore, that when a sufficient quantity of killed cultures of *Leptospira icterohæmorrhagiæ* is given, the guinea pigs will become immune to all strains of the same organism, but that smaller quantities may protect them against homologous but not against heterologous strains. A close analysis reveals the existence of group or type affinities among different strains which can be brought out by immunizing the animals with smaller quantities of killed cultures. In the present series of experiments American Strains 1 and 3 form one group, American Strain 2 and the European strain another, and the Japanese strain a third, which is also closely allied to the first group.

In order to insure universal immunity it is wise to employ as many group or type cultures as possible in the preparation of vaccines, a polyvalent vaccine being recommended. It is not improbable that the strain recently encountered in Lorient, France,[5-8] is an unusually deviated type of *Leptospira icterohæmorrhagiæ*, and that if successfully cultivated and used as vaccine in sufficient amount it might protect the animals against other strains of the same organism.

The active immunity induced in the vaccinated guinea pigs was found to persist for at least 8 weeks after the last inoculation. It will no doubt last for a much longer period.

[5] Manine, Cristau, and Plazy, *Compt. rend. Soc. biol.*, 1917, lxxx, 531.

[6] Manine and Cristau, *Bull. et mém. Soc. méd. hôp. Paris*, 1917, xli, series 3, 977, 1045.

[7] Pettit, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 774.

[8] Pettit, A., *Compt. rend. Soc. biol.*, 1918, lxxxi, 48.

[Reprinted from The Journal of Experimental Medicine, November 1, 1918,
Vol. xxviii, No. 5, pp. 629–644.]

EXPERIMENTAL HEMOCHROMATOSIS.

By PEYTON ROUS, M.D., and JEAN OLIVER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 64 to 70.

(Received for publication, June 17, 1918.)

In pernicious anemia and hemochromatosis an iron-containing pigment, hemosiderin, ordinarily derived from hemoglobin, is deposited in organs free from such pigment in most conditions that involve blood destruction. When sudden hemolysis takes place in a previously healthy human being, as after cutaneous burns or the action of a "blood poison," hemosiderin granules appear in the spleen, the red marrow, and sometimes in certain lymph nodes, but as a rule in these organs only. A similar localization of the pigment is found in most diseases accompanied by a destruction of red cells,—acute malaria, for example; and it is regularly encountered in animals submitted to experiments or infections which, like trypanosomiasis, involve a breaking down of blood. Far different is the siderosis of pernicious anemia and hemochromatosis. The spleen and bone marrow are still pigmented, but to only a slight degree as compared with some of the other organs. In pernicious anemia most of the hemosiderin is found in the liver and much in the kidneys. In hemochromatosis the liver, kidneys, pancreas, heart, abdominal lymph nodes, and many other organs are heavily loaded with the pigment.

What is the cause for the difference thus outlined? Has the hemosiderin in hemochromatosis another source than the blood? These and other important questions suggest themselves. And numerous attempts to answer them through an experimental production of the significant lesions have already been made. Stadelmann,[1] Hunter,[2] Biondi,[3] and others obtained a siderosis of the liver parenchyma as the

[1] Stadelmann, E., *Arch. exp. Path. u. Pharmakol.*, 1887, xxiii, 427.
[2] Hunter, W., Pernicious anæmia, London, 1901, 182.
[3] Biondi, C., *Beitr. path. Anat. u. allg. Path.*, 1895, xviii, 176.

result of fatal doses of toluylenediamine. The drug injures the liver greatly. Schurig,[4] who injected pure hemoglobin into rabbits day after day for several weeks found some hemosiderin in the liver and kidneys, in addition to the usual abundance in spleen and marrow. Muir and Dunn,[5] producing an acute hemolytic anemia in rabbits by means of a specific hemolysin, noted a little granular hemosiderin in the liver and a diffuse Prussian blue reaction in the kidney parenchyma of animals surviving the destruction in the course of 3 days of more than half their total blood. Other less successful results might be quoted. The general findings warrant the conclusion that under most circumstances of blood destruction the spleen and red marrow act as depots for hemosiderin and more than suffice for the purpose; but when the breaking down of red cells is fulminant, or large quantities of blood pigment are furnished the organism for a considerable period, the buffer activity of these organs is overcome, and some little hemosiderin is laid down elsewhere. There are several obstacles to obtaining a great deposition of it by experimental means. Free hemoglobin injected in considerable amount is for the most part rapidly lost through the kidneys; blood cells that are damaged tend to accumulate in the capillaries and cause lesions; while the blood poisons, repeatedly administered, injure other tissues besides the blood, with complex results. In view of these circumstances the unsatisfactory outcome of experiments is easily understood.

Method.

It has seemed to us that better results than those described might be obtained through a utilization of the animal's own ability to break down blood under circumstances such that more pigment is liberated than spleen and marrow can deal with. A means for the work has come to hand in the method of repeated transfusion employed by one of us with Robertson for the study of normal blood destruction.[6] Rabbits rendered plethoric by the day to day injection of 10 cc. of the citrated whole blood of other rabbits soon acquire the ability to dispose

[4] Schurig, *Arch. exp. Path. u. Pharmakol.*, 1898, xli, 29.

[5] Muir, R., and Dunn, J. S., *J. Path. and Bacteriol.*, 1914–15, xix, 417.

[6] Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1916, xxv, 665.

rapidly of the foreign blood, and this in many instances without the development of demonstrable immune bodies. We have accordingly transfused eleven animals for months, injecting them 6 days out of every 7, and towards the end increasing the daily amount of blood to 15 cc. For each recipient a number of donors were used in rotation. In the early weeks some of the recipients developed weak agglutinins for the foreign blood. Later, as the injections were continued, such antibodies disappeared.

Findings in Relation to Pernicious Anemia.

In the present paper a general description of the results of the work will be given. A study of certain of its special features is to be published later.

The rabbits killed after a few weeks of plethora induced by the repeated injection of blood showed merely the lesions already described by many observers[7] as following one or two massive transfusions; namely, an enlarged spleen, turgid with cellular debris and pigment, and phagocytes full of red cells; a considerable siderosis of the marrow; and a slight one of the abdominal lymph nodes—in other words, the findings usual after blood destruction in normal animals. When the injections had been kept up for 2 months there was in addition a pigmentation of the parenchyma of the liver and kidneys with fine yellow-brown granules which on test proved iron-containing. The pigment was abundant in the peripheral cells of the hepatic lobules and in the convoluted tubules of the kidneys, as is the case in pernicious anemia. Save for the siderosis, a general plethora, and a consequent slight, central atrophy of the liver cells, the organs appeared normal. In human pernicious anemia the spleen is relatively free from pigment, whereas in our animals this organ was always most heavily loaded with it. The difference is an important one, indicating as it does either that the spleen is altered in pernicious anemia, and unable to take up the products of blood destruction, or that its activity is somehow circumvented, which is the supposition in Hunter's theory of a portal blood destruction.

[7] For instance, Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1910, xiv, 294.

Findings in Relation to Hemochromatosis.

Seven rabbits were transfused over a period of from 3 to $6\frac{1}{2}$ months. The findings in these have great interest, for they are such as are associated, in human beings, with hemochromatosis and with this disease alone. Most of the animals were healthy when killed and had gained weight despite the long standing plethora which involved a day to day hemoglobin of 120 to 160 per cent Sahli, and, as the autopsies showed, a pronounced general congestion. Jaundice was not noted at any time, nor was hemoglobin or sugar found in the urine. The absence of the latter is accounted for by the state of the pancreas which showed lesions of a mild grade in contrast to the serious changes which accompany the *diabète bronzé* of man.

In comparing the rabbit material with that of human hemochromatosis, we have made use of the recent excellent descriptions of Sprunt,[8] Gaskell and his associates,[9] and also of specimens from an outspoken case of hemochromatosis studied at the Hospital of The Rockefeller Institute, and autopsied by one of us.[10] The older studies of hemochromatosis are in general marred by the use of unsatisfactory methods to demonstrate iron-containing pigment.

The changes in the rabbits were, in general, much less marked than those observed post mortem in human hemochromatosis, as would naturally follow from the fact that the pigmentary process had not gone so far in the animals as even to menace health. Nevertheless, many of their organs, notably the liver, kidney, heart, visceral lymph glands, pancreas, spleen, and marrow, had, to the naked eye, a distinct brown tint. The liver was in several instances of a light chocolate color and showed a slight, diffuse cirrhosis, indistinguishable in the gross from that so frequently intercurrent in rabbits. The spleen was never larger than after several weeks of transfusion and often smaller. The skin of a long transfused white rabbit had a brownish tone. In every case the vessels were engorged, the liver was swollen, owing to congestion, and the heart was enlarged and dilated. The histological findings were essentially similar in all the animals, differing only in degree.

[8] Sprunt, T. P., *Arch. Int. Med.*, 1911, viii, 75.

[9] Gaskell, J. F., Sladden, A. F., Wallis, R. L. M., Vaile, P. T., and Garrod, A. E., *Quart. J. Med.*, 1913–14, vii, 129.

[10] Rous, P., *J. Exp. Med.*, 1918, xxviii, 645.

Pigments.

Most of the pigment in the organs differed not at all in appearance or reactions from the hemosiderin observed in human beings. It had the form of orange-brown, moderately refractile granules, usually minute, but sometimes coarse and irregular, as in the spleen. When exposed to ammonium sulfide the granules turned black, and when tested by Nishimura's method[11] they took on a fine, deep blue color. In determining their distribution to the various organs, we have regularly used the latter method, which Sprunt and others, working with human material, have found satisfactory.

Hemofuscin, a yellow or brown pigment which fails to react for iron, occurs in quantity in some cases of human hemochromatosis, whereas in others it is practically absent. As Sprunt has pointed out, its amount varies greatly with the method used to demonstrate it. With the Nishimura test there is always far less than with Perl's reaction which the older authors generally employed for the demonstration of iron. The hemofuscin is to some extent characterized by its situation, being found especially in the capsules and trabeculæ of lymph nodes and in the smooth muscle of the walls of blood vessels. Very little was present in the numerous preparations from our recent case of human hemosiderosis. In the transfused rabbits a yellow pigment which failed to respond positively to the Nishimura test was observed in scattered connective tissue cells of the lungs and choroid plexus.

Comparison of the Human and Rabbit Organs.

Spleen.—The condition of the spleens of rabbits long transfused justifies the conclusion already expressed that this organ serves as a buffer depot for hemosiderin, and that its activity in this direction must be overcome if the pigment is to reach the other viscera in quantity. In animals killed after only 3 to 4 weeks of plethora the spleen was much enlarged, turgid with cellular debris and with phagocytes crowded with red cells. The liver and other viscera were at this period practically non-pigmented. But when the plethora had been maintained for months an entirely different state of affairs was found. The spleen, no larger than before, and often rather small, was a mere

[11] Nishimura, J., *Centr. allg. Path. u. path. Anat.*, 1910, xxi, 10.

congeries of sinuses, containing large, irregular masses of hemosiderin, and distended with blood, but almost empty of other cells (Fig. 1). The Malpighian corpuscles were atrophic, and many had nearly disappeared. The organ was, in fact, a mere shell, scarcely to be thought of as functioning longer for the retention of blood pigment. And, as would naturally follow from this fact, the liver and many other organs now showed profuse deposits of hemosiderin.

In the hemochromatosis of man there is chronic passive congestion of the spleen consequent on the liver cirrhosis characteristic of the disease. Phagocytes distended with hemosiderin are present in moderate number, but the greater part of the pigment, which at most is not abundant as compared with the amount in other organs, lies in the reticular cells. The Malpighian bodies are not atrophied. A diffuse increase in the connective tissue may usually be noted. There is no sign that a great activity of the organ to store up pigment was once present and has been overcome.

Bone Marrow.—The marrow of the rabbit, after months of plethora from transfusion, showed little more pigment than after a few weeks (Fig. 2). Evidently the limit of its capacity for the reception of hemosiderin had soon been reached. The greater part of the pigment lay in distended mononuclear cells. The entire quantity was only moderate. In man, too, this is the case. No distinct changes in the blood-forming tissues are to be noted in either animal.

Liver.—In the rabbits this was, after the spleen and certain of the abdominal lymph nodes, the most heavily pigmented organ. In one instance the degree of siderosis approached that in some patients dying of hemochromatosis (Fig. 3). The parenchyma contained everywhere numerous pigment granules but notably at the periphery of the lobules, where often the cells were filled with coarse yellow lumps that turned deep blue when tested by Nishimura's method. Similar iron-containing pigment lay in the capillaries, much of it in great rounded masses enclosed more or less completely in multinucleate giant cells. Many Kupffer cells were filled with pigment granules. Towards the center of the lobules the capillaries were widely distended, without doubt as a result of the plethora, and there was some pressure atrophy of the otherwise normal looking parenchymal elements. In two of the most pigmented livers there was some intralobular cirrhosis, but in a

third this element was quite lacking, and careful study has convinced us that such cirrhosis as was found was intercurrent in character, of a sort frequently seen in supposedly normal rabbits. The question of its relationship to the pigmentation will be discussed in detail further on. The cirrhotic tissue contained some new-formed bile ducts, but neither these nor the normal ones were pigmented, though here and there a connective tissue cell was laden with the characteristic brown granules.

In man the siderosis of the liver parenchyma has the same general appearance and an identical distribution with that in the rabbit, being more abundant towards the periphery of the lobules. Here many of the parenchymal cells may become so filled with pigment as to break down, resulting in connective tissue proliferation and the appearance of islands of new-formed liver cells. There is regularly present a pronounced hypertrophic cirrhosis with many new-formed and heavily pigmented bile ducts. This characteristic feature of the human disease was absolutely lacking in our rabbits.

Kidneys.—There was in the rabbits a marked siderosis of the kidney parenchyma (Fig. 4) somewhat more general in its distribution than is the case in human hemochromatosis. In this latter disease the pigment is, as a rule, sharply localized to the ascending limb of Henle's loop and to the distal convoluted tubules, where it is abundant; while the rest of the parenchyma, save in some cases the glomeruli, is free from it. Gaskell and his associates[9] point out that in pernicious anemia, by contrast, the pigment is found almost wholly in the proximal convoluted tubules. In our rabbits the glomeruli never showed pigment, but the cells of nearly all the cortical tubules contained it. Very fine granules were present in moderate number in the proximal convoluted tubules; more were to be seen in the descending limb of Henle's loop; and the ascending limb and distal convoluted tubules were very heavily pigmented with coarse, granular agglomerates, just as in the hemochromatosis of man. And, as in this disease, the collecting tubules and medulla were free from pigment.

Pancreas.—Only in the animals transfused during a long period was this organ pigmented. Fine hemosiderin granules were then demonstrable in the alveolar cells, often in considerable quantity (Fig. 5). The islands of Langerhans were always normal, and there was an en-

tire absence of the cirrhosis which in human beings may be marked. But there was an absence too of the destruction of gland tissue through excessive pigmentation which many authors hold to be the cause of the cirrhosis.

Adrenals, Stomach, and Intestines.—In two rabbits the adrenal glands showed a slight siderosis and in precisely the region where pigmentation is found in man; namely, in the zona glomerulosa of the cortex (Fig. 6). The intestines, as in the human instance, were free from hemosiderin save for the granules in a few connective tissue cells scattered throughout the coats. But the stomach also was unpigmented whereas in the human stomach the glands of the mucosa are usually the subject of an outspoken siderosis, especially at their base.

Heart.—This organ showed pigment only in the rabbits that were transfused longest. The hemosiderin was deposited in fine granules throughout the muscle fibers, being especially abundant close to either end of the nucleus (Fig. 7). An identical localization of the pigment is found in human beings, but the amount is, as a rule, far greater, sometimes leading to death of the muscle fibers with connective tissue overgrowth as a further result.

Lungs.—Neither in man nor in the rabbit is there a noteworthy pigmentation of the lungs. A few iron-containing cells may be present here and there in the reticulum and sometimes in the capillaries.

Lymph Nodes.—In the rabbits with most advanced siderosis large masses of free iron-containing pigment were present in the sinuses of the lymph nodes draining the liver (Fig. 8) and granules were present in many cells, as well. The other abdominal lymph nodes also contained iron in some quantity, but so sometimes do those of the normal rabbit. The mediastinal lymph glands of the transfused rabbits and those from the groins and axillæ showed at most only a few granules. This localization of hemosiderin to the nodes draining heavily pigmented organs is the rule in human hemochromatosis.

Skin.—The bronzed portions of the human skin usually contain slaty brown pigment granules in the basal layers of the epithelium, which do not react positively to the tests for iron. Ordinary hemosiderin is present as well in connective tissue cells of the corium, especially about the sweat glands. In our rabbits the epidermal pigment was lacking, but connective tissue cells containing hemosiderin were often fairly numerous in the corium (Fig. 9).

Other Organs.—In one of Sprunt's cases the tracheal cartilage was pigmented and in another the submaxillary gland. Both these lesions have been found in our rabbits. Siderosis of the submaxillary gland was well marked in one animal, the granules lying grouped in the alveolar cells (Fig. 10). The rabbit thyroid, on the other hand, was never affected, whereas in man it may be loaded with pigment. The rabbits transfused longest happened to be old females. In them a pronounced siderosis of the mammary gland was regularly present, certain cells of the epithelium being distended with pigment, while still others containing it had desquamated and could be found free in the lumen of the ducts (Fig. 11). There is no parallel in man for this lesion, since human hemochromatosis is a disease of the male. Abbott's description of the single case on record in a female does not include a report on the mammary gland.

Relation of Cirrhosis and Pigmentation.

Hypertrophic cirrhosis of the liver is a constant feature of human hemochromatosis, but whether it is a primary or secondary factor in the disease has not been determined. There is no doubt that a fibrous overgrowth is often present that has been induced by parenchymal destruction. The evidence of such a secondary connective tissue proliferation is so clearly visible in autopsy material that many authors have been led to consider the entire cirrhosis as secondary. But actually the recognition of areas of induced cirrhosis proves nothing either way, since they would be present in any event were the pigmentation sufficient to cause parenchymal destruction.

The transfused rabbits were killed at a relatively early stage of hepatic siderosis, and the ability of this latter to induce cirrhosis in the absence of cell destruction can be well judged from the findings. At the periphery of the lobules the parenchymal cells were heavily pigmented, but as yet only here and there had one broken down. Cirrhosis was practically absent, save for that of an intercurrent nature, which at most was slight, and obviously it had not followed the pigmentation, being often well marked where the latter was negligible. Here and there in the lobular capillaries were great masses of iron pigment, some of them as much as 80 μ across, more or less completely enclosed in multinucleate giant cells. But though these pigment

masses had induced pressure atrophy of the surrounding parenchymal cells, no connective tissue reaction had taken place about them. It may be urged that proliferation might have ensued in time, and this is not impossible. Yet in view of the lack of an immediate reaction the conclusion seems justified that hemosiderin when enclosed in living cells has no noteworthy stimulative effect on connective tissue.

Hepatic changes similar in degree to those in the rabbits cannot be expected at the average human autopsy, since patients with hemochromatosis do not, as a rule, succumb to the disease until the pigmentation has destroyed large numbers of parenchymal cells with a resulting connective tissue overgrowth. Only by some chance may one expect to obtain the liver at an earlier stage. Such a chance has recently come to us in the case of hemochromatosis already mentioned. The patient died, not of this disease, which apparently had lasted about $1\frac{1}{2}$ years, but of an old chronic myocarditis.[10] The cells at the periphery of the liver lobules were pigmented only to the degree found in rabbits and showed no evidence of injury. But here the parallel ceases. For whereas in the rabbits an hepatic cirrhosis was absent, in the human liver a strikingly abundant one was found, interlobular, and of long standing, as shown by its character. Save in the cells of the many new-formed bile ducts, the cirrhotic tissue contained almost no pigment. That the connective tissue overgrowth was in this instance the result of parenchymal destruction from excessive pigmentation is out of the question. One is led inevitably to conclude that it must have been a primary feature of the patient's disease.

If the fact is granted, as the evidence warrants, that cirrhosis is a primary occurrence in some cases at least of hemochromatosis, this does not mean that it is without a relation to the characteristic pigmentation. Kretz[12] has shown that cirrhosis of any sort is frequently accompanied by a more or less marked siderosis of the liver. He found such a pigmentation in fourteen out of twenty-six cirrhotic livers, whereas in only one of every fifteen or twenty that were non-cirrhotic was it present. The rabbit material has furnished striking proof that when a tendency to siderosis exists an intercurrent cirrhosis will

[12] Kretz, R., *Centr. allg. Path. u. path. Anat.*, 1897, viii, 620.

greatly increase the deposition of pigment. In one transfused animal with a very moderate general siderosis of the liver an adhesion had occurred connecting the anterior edge of a lobe with the abdominal wall and thus inducing a well marked local cirrhosis. Here the liver cells were so heavily pigmented as in many instances to be scarcely recognizable and in others to have broken down (Fig. 12). The contrast to the parenchyma elsewhere in the organ was extreme. In a second animal, as well, a local cirrhosis of unknown origin was accompanied by great local pigmentation. It is perhaps not a matter of accident that the rabbit with the most pigmented liver had not been transfused longest but was the subject of an intercurrent cirrhosis. In the course of 167 days it had received 139 transfusions—52 of 10 cc. each and the last 87 of 15 cc. each. A companion animal of the same weight and sex, receiving in 194 days 156 transfusions, the last 99 of 15 cc. each, had no cirrhosis of the liver and exhibited by contrast little pigmentation.

All this gives good reason for the supposition that the pigmentation of the liver cells in human hemochromatosis is in large part, if not entirely, secondary to the cirrhosis. Whether the connective tissue overgrowth of the pancreas that is so often observed is an essential phenomenon of the human disease or is secondary to the abundant parenchymal destruction, our work does not enable us to say. But a similar fibrous overgrowth, which is obviously of secondary character, may often be observed in the heavily pigmented heart of hemochromatosis cases, and sometimes in the thyroid gland.

Injury and Pigmentation.

Signs of an old perirenal inflammation existed in one of the transfused rabbits, and in the scar tissue here numerous connective tissue cells were noted containing hemosiderin. The observation led us to inject agar into the other animals of the series some days before killing them, with a view to determining whether iron would be laid down in the reactive tissue. Such was the case. The cells in the interior of the agar mass were unpigmented, which was to have been expected, since they were in process of rapid proliferation, but in the older reactive tissue hemosiderin was fairly abundant as small granules in the fibro-

blasts (Fig. 13). Its constant presence and general distribution rules out the possibility that it was derived from local hemorrhages.

A similar relation of injury to pigmentation was observed in the human instance of hemochromatosis already several times cited. The arteries showed scleroses here and there. In the sclerotic patches iron was sometimes encountered in quantity (Fig. 14), whereas in the healthy arterial wall it was entirely lacking. The ability of hepatic cirrhosis to induce pigmentation is, of course, only another example of the effect of injury, but of injury this time to an organ essentially concerned in the degradation of blood pigment.

A simple explanation now suggests itself for the curious distribution of the cutaneous pigmentation in human hemochromatosis. The face and neck to the collar line, and the backs of the hands are the surfaces which become most bronzed and are often the only ones affected. These are precisely the surfaces most subjected to light, wind, and other injurious influences, such as may be supposed from the evidence just given to conduce to a deposition of pigment. There is, however, no gainsaying the fact that not every sort of injury can bring about cutaneous siderosis, else one would expect it on the soles of the feet, for example. But the data given by Kretz show that even in the liver the injury must be of a special sort if iron is to be laid down. He has pointed out that neither the liver degenerations which result from phosphorus and arsenic poisoning, nor chronic passive congestion, nor cholelithiasis with jaundice suffices to cause the least hepatic siderosis.

An Aid to Diagnosis.

Hemosiderin granules were frequently found lying free in the lumen of the kidney tubules of the heavily pigmented rabbits, and they were also noted embedded in casts (Fig. 4). The fact suggested that in human beings a diagnosis might be made through the demonstration of hemosiderin granules in the urine. This would be far preferable to the excision of a piece of skin, the procedure usual at present. A diagnosis has been accomplished by the method, as is shown in another paper.[10]

On the Sequence of Events in Human Hemochromatosis.

The hemochromatosis of transfused rabbits, as thus far studied, has points of difference from the human disease, but also important points in common with it. The points of difference are in large part those of degree and of time. For instance, none of the animals developed diabetes; but for that matter in none was the pancreas greatly damaged as it is in *diabète bronzé*. The pigmentation of the pancreas, as far as it went, was like that occurring in man. This example well illustrates the general variances and likenesses found. The pigmentation of the rabbits was strikingly similar to that in human beings, not merely in kind but in distribution to the organs and in them. The sole difference in the pathological findings which must be considered as essential was the total absence in rabbits of the hepatic cirrhosis which in man is a constant and pronounced feature of hemochromatosis. There are good reasons, already stated, for the belief that this cirrhosis is a primary factor in the disease.

Most of the recent evidence in the literature is against the older view of excessive blood destruction in an otherwise healthy organism as the cause of hemochromatosis. So too are our experiments with rabbits. The transfused animals had disposed of immense quantities of blood before any noteworthy pigmentation appeared. Some of them received in the course of each week during a long period an amount of blood almost as great as their own original total quantity (5.5 per cent of the body weight).[13] The organism was required to dispose of this and of its own worn out corpuscles as well, yet at most the pigmentation was moderate compared with that found in man. Needless to say this excessive and continued blood destruction could not be supported by the human body without notable changes in the circulating red cells and the blood-forming organs, and these are completely absent.

While an increased destruction of red cells cannot be the primary cause of hemochromatosis, yet certainly one need look no further than these elements for the source of the hemosiderin. The presence of the pigment in such peculiar situations as the heart muscle and the parenchyma of many glands has led some authors to suppose that there must

[13] Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1909, xiii, 256.

be a derangement of these tissues, causing them to elaborate hemosiderin out of their own proper substance. But in our rabbits the pigment laid down was manifestly attributable to erythrocytes undergoing destruction, and it was found in precisely the same situations as in human hemochromatosis.

The activity of the liver is known to be profoundly important to the working over of blood pigment. The sequence of events in human hemochromatosis would seem to be the following: A liver cirrhosis of unknown origin leads to a failure of the organ to deal adequately with the iron-containing products of normal blood destruction, and the latter accumulate in the organism. Widespread pigmentation results automatically. It at length becomes so great as to cause the death of parenchymal cells, especially in the liver and pancreas, thus leading to secondary connective tissue overgrowth. This, in turn, through injury to the remaining parenchyma, hastens the accumulation of pigmentation. By a repetition of the vicious circle what is essentially a chronic disease becomes at length a rapid progress down-hill. Diabetes appears in many cases as a result of the pancreatic injury, and soon death results. There is no link in this chain of events for which the facts do not afford strong evidence.

SUMMARY.

In rabbits destroying transfused blood constantly during a period of many months a pronounced and widespread siderosis ensues, practically identical with that characterizing human hemochromatosis. The findings do not indicate the ultimate cause of this disease, but they throw light on its various features and its course, and suggest a means for its diagnosis.

EXPLANATION OF PLATES.

The sections from which Figs. 1 and 8 are taken were stained with methylene blue and eosin. Nishimura's method was used with all the other material to demonstrate iron-containing pigment, and lithium carmine was employed as a counterstain.

PLATE 64.

FIG. 1. Spleen of a rabbit (A) transfused 139 times during a period of 167 days. The sinuses are distended with blood. An atrophic Malpighian corpuscle may be

seen near the center of the photograph. The black masses consist of hemosiderin, for the most part extracellular.

FIG. 2. Bone marrow of the same animal. The hemosiderin appears black, as is the case also in the photographs which follow.

PLATE 65.

FIG. 3. Liver of the same animal as in Figs. 1 and 2. The cirrhosis is intercurrent. The iron is especially abundant towards the periphery of the lobules, but the parenchyma shows granules of it everywhere.

FIG. 4. Kidney of a rabbit (B) transfused 120 times in the course of 180 days. The glomeruli are free from pigment, but it is present in all the tubules except the collecting ones, being especially abundant in the ascending limb of Henle's loop and in the distal convolutions. A cast is to be seen containing many fine hemosiderin granules.

PLATE 66.

FIG. 5. Pancreas of a rabbit (C) transfused 156 times in the course of 194 days. The alveolar cells towards the center of the photograph contain hemosiderin in especially large amount.

FIG. 6. Iron-containing pigment in the peripheral cells of the zona glomerulosa of the adrenal gland. Rabbit C. The connective tissue capsule of the organ occupies the upper part of the photograph.

PLATE 67.

FIG. 7. Heart muscle of Rabbit A. The hemosiderin is especially abundant at either end of the nuclei of the muscle fibers.

FIG. 8. Section of a lymph gland draining the liver of Rabbit A. Large aggregates of extracellular hemosiderin are to be seen.

PLATE 68.

FIG. 9. Skin from the abdomen of Rabbit B. Iron-containing pigment is present in scattered cells of the corium.

FIG. 10. Hemosiderin granules in the alveolar cells of a submaxillary gland. Rabbit C. The patchy localization here seen was present throughout the gland.

PLATE 69.

FIG. 11. Mammary gland of Rabbit A, an old female. Many of the heavily pigmented cells have desquamated. The animal had not been pregnant during the period of transfusion.

FIG. 12. Heavy pigmentation secondary to a local cirrhosis of the liver. Rabbit B. Non-cirrhotic tissue may be seen at the lower left hand corner of the photograph. Here the pigmentation of the parenchyma is by contrast very slight.

PLATE 70.

FIG. 13. Hemosiderin in cells of the reactive tissue surrounding a mass of agar-agar. Rabbit B. The clear material is the agar, which is in process of organization. Iron-containing pigment is present only in the older scar tissue, not where the cells are actively proliferating.

FIG. 14. Sagittal section of a small artery from the patient with hemochromatosis. Iron-containing pigment has been deposited where the media is degenerated. The rest of the vessel wall is free from it.

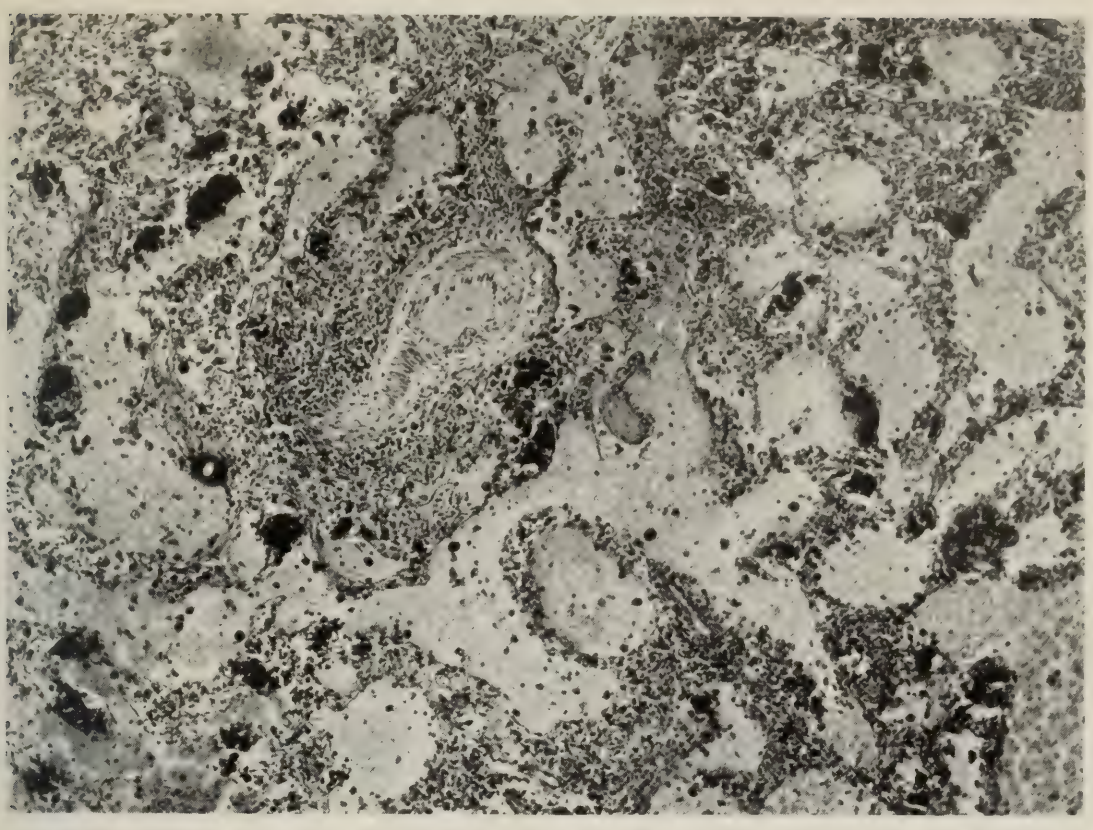


FIG. 1.

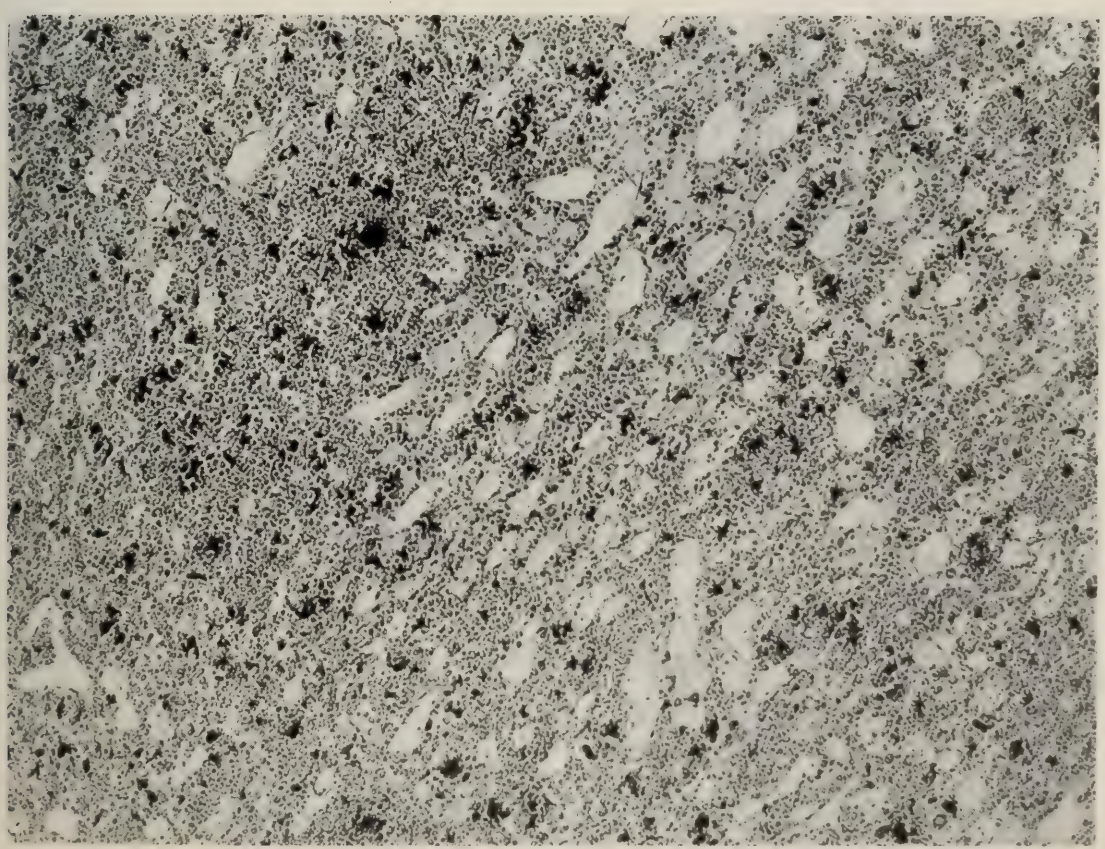


FIG. 2.

(Rous and Oliver: Experimental hemochromatosis.)

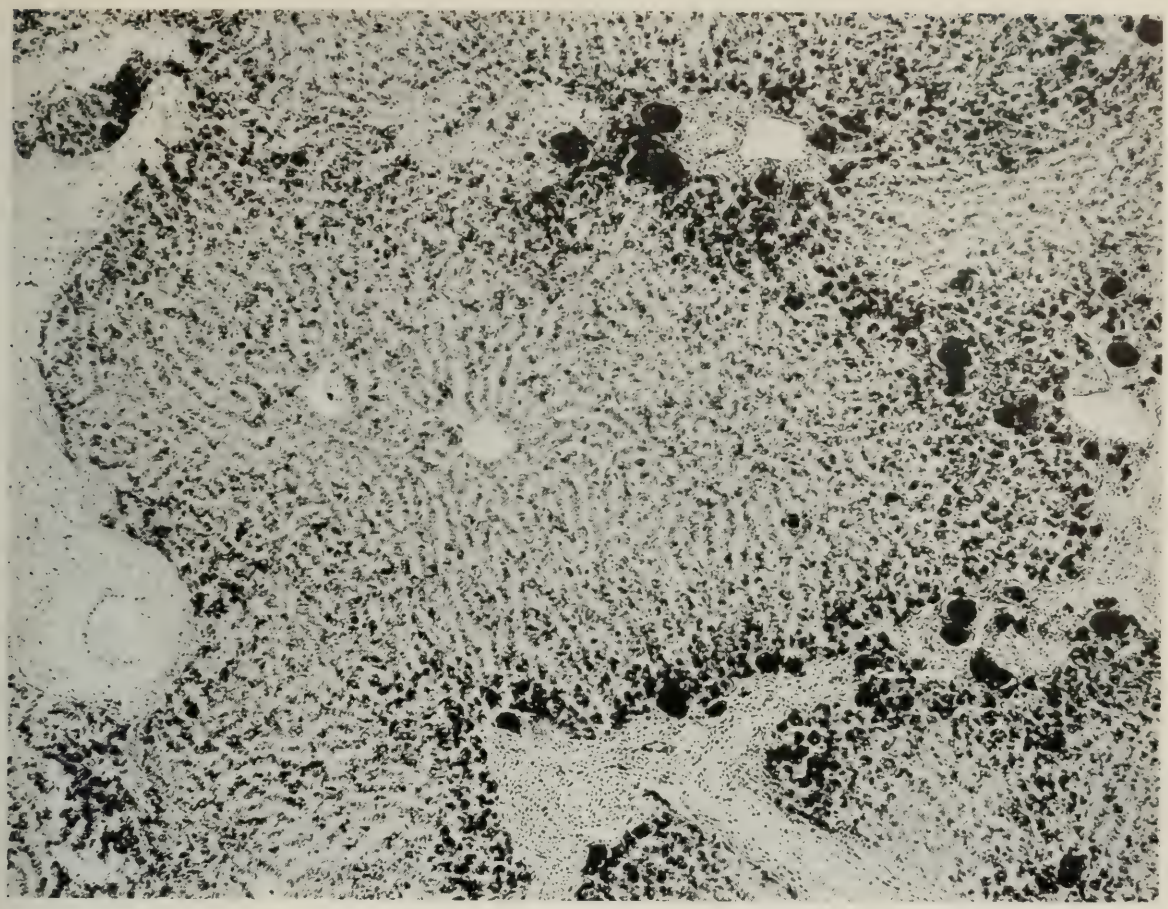


FIG. 3.

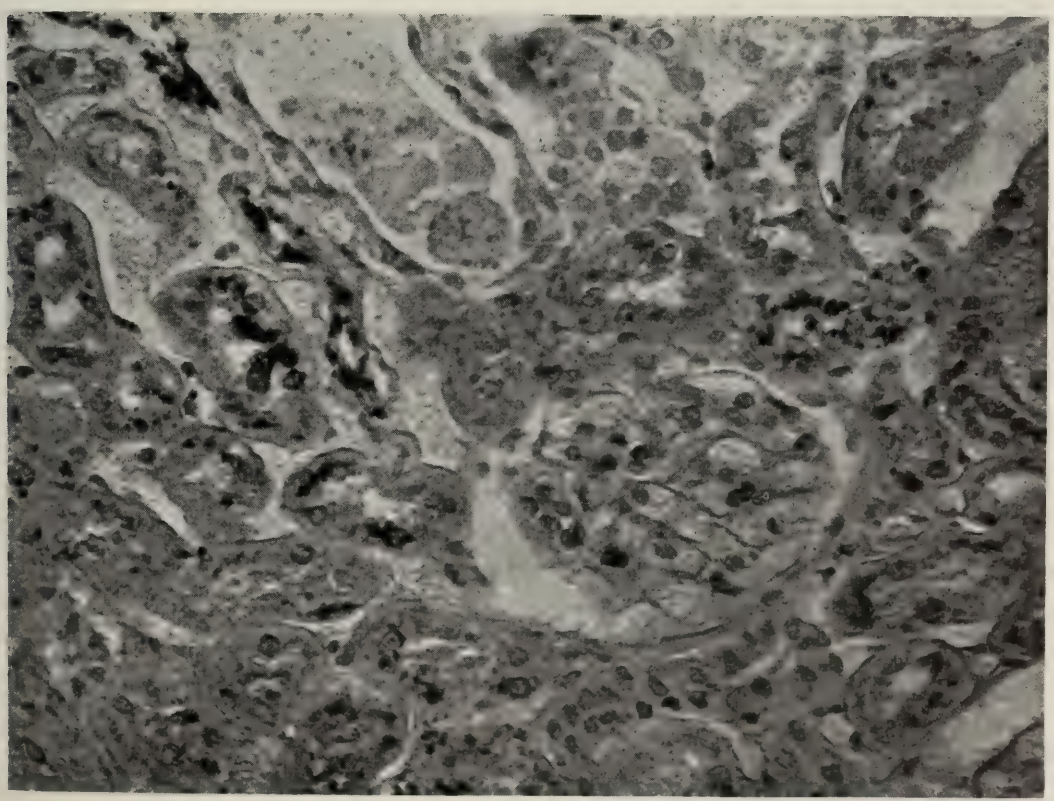


FIG. 4.

(Rous and Oliver: Experimental hemochromatosis.)

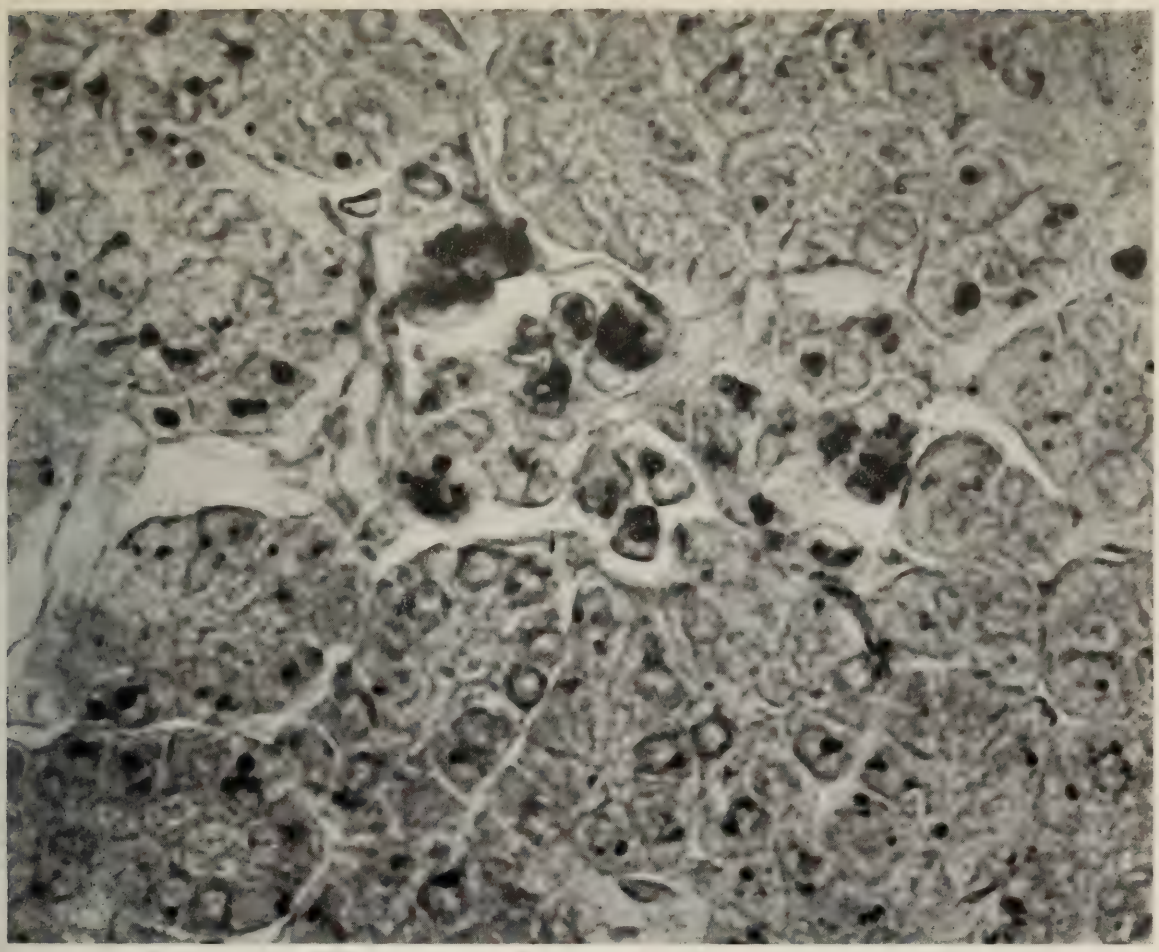


FIG. 5.

FIG. 6.

(Rous and Oliver: Experimental hemochromatosis.)

FIG. 7.

FIG. 8.

(Rous and Oliver: Experimental hemochromatosis.)

FIG. 9.

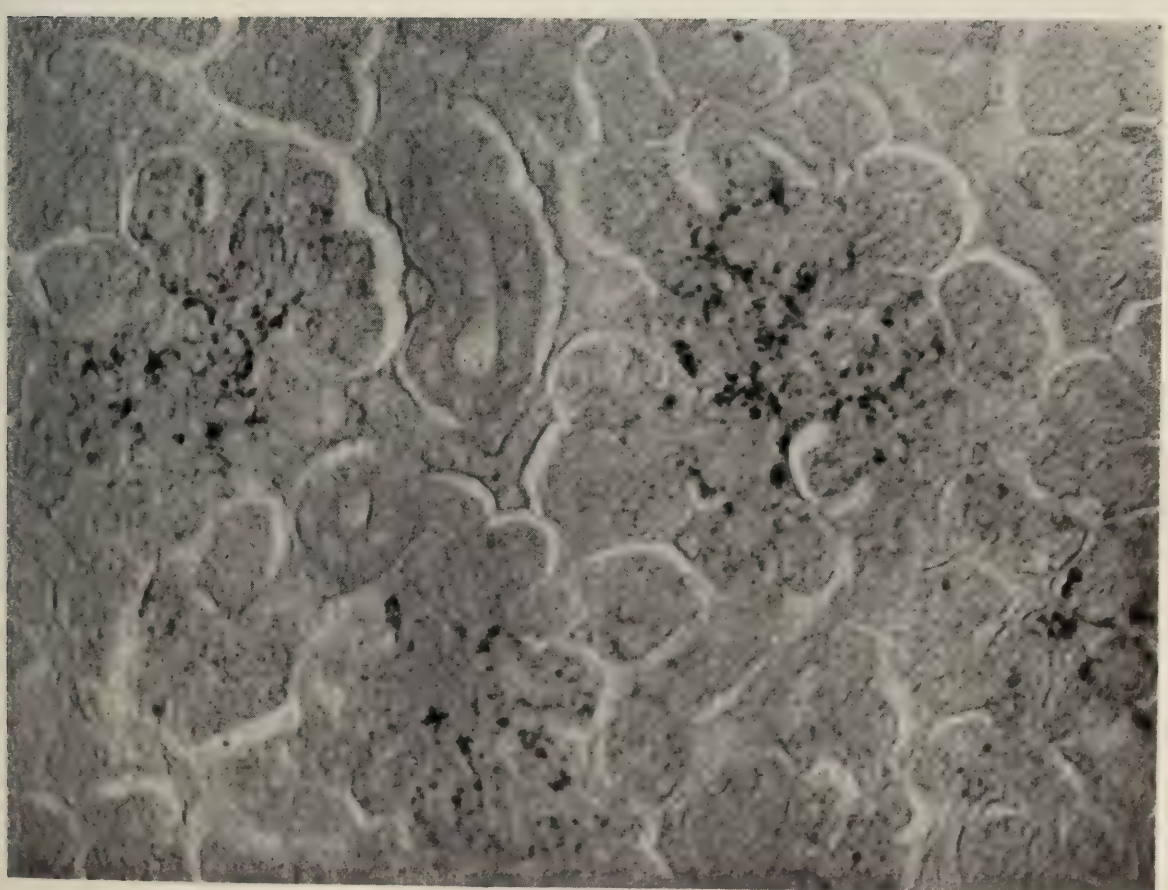


FIG. 10.

(Rous and Oliver: Experimental hemochromatosis.)

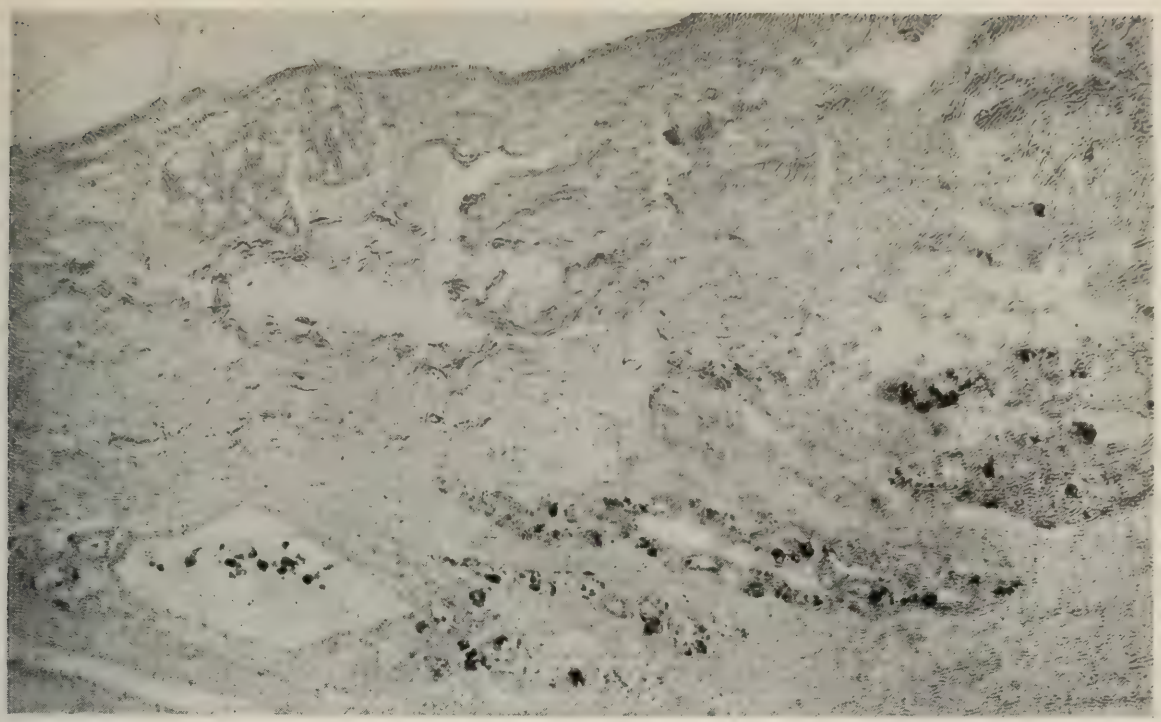


FIG. 11.

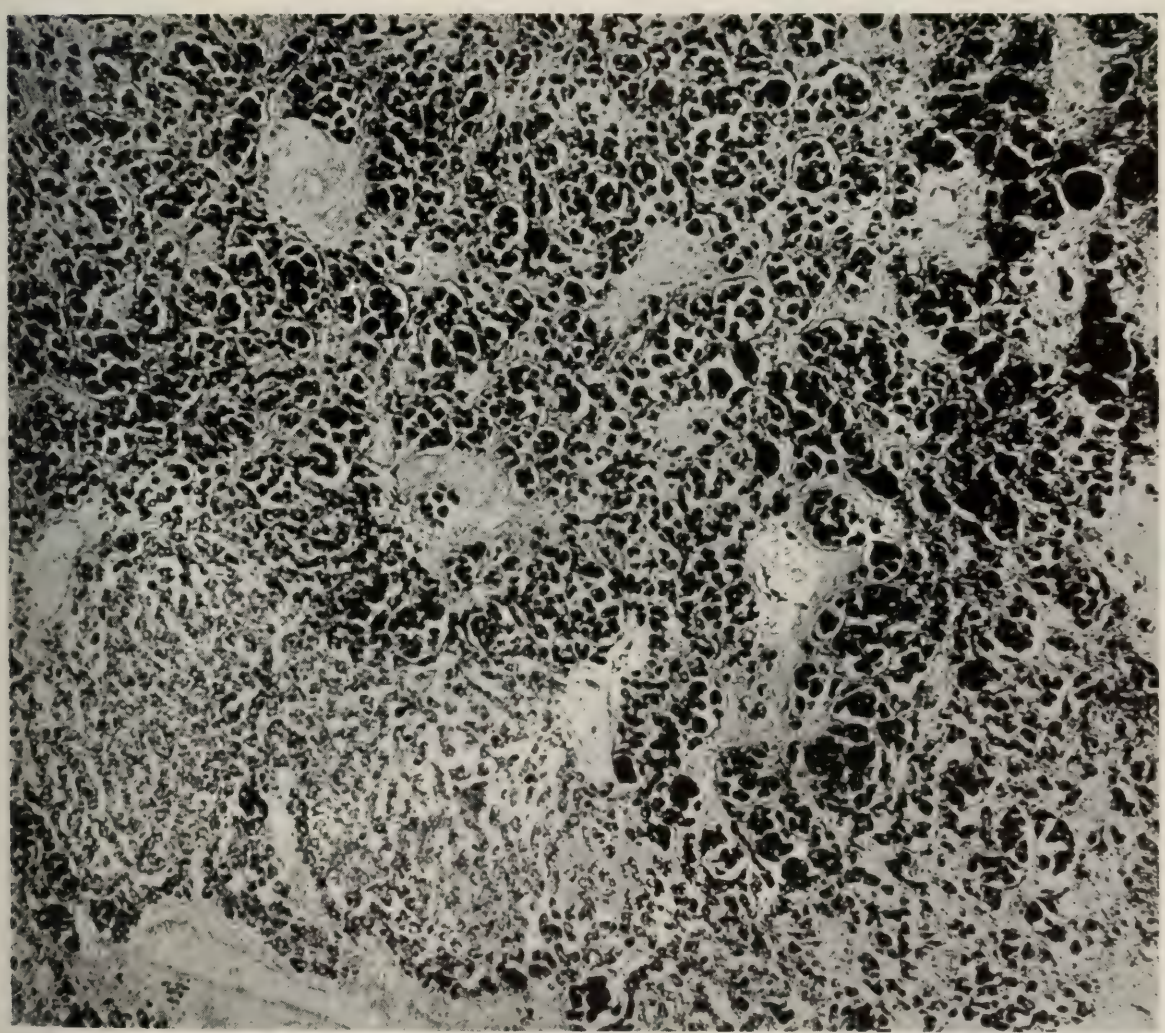


FIG. 12.

(Rous and Oliver: Experimental hemochromatosis.)

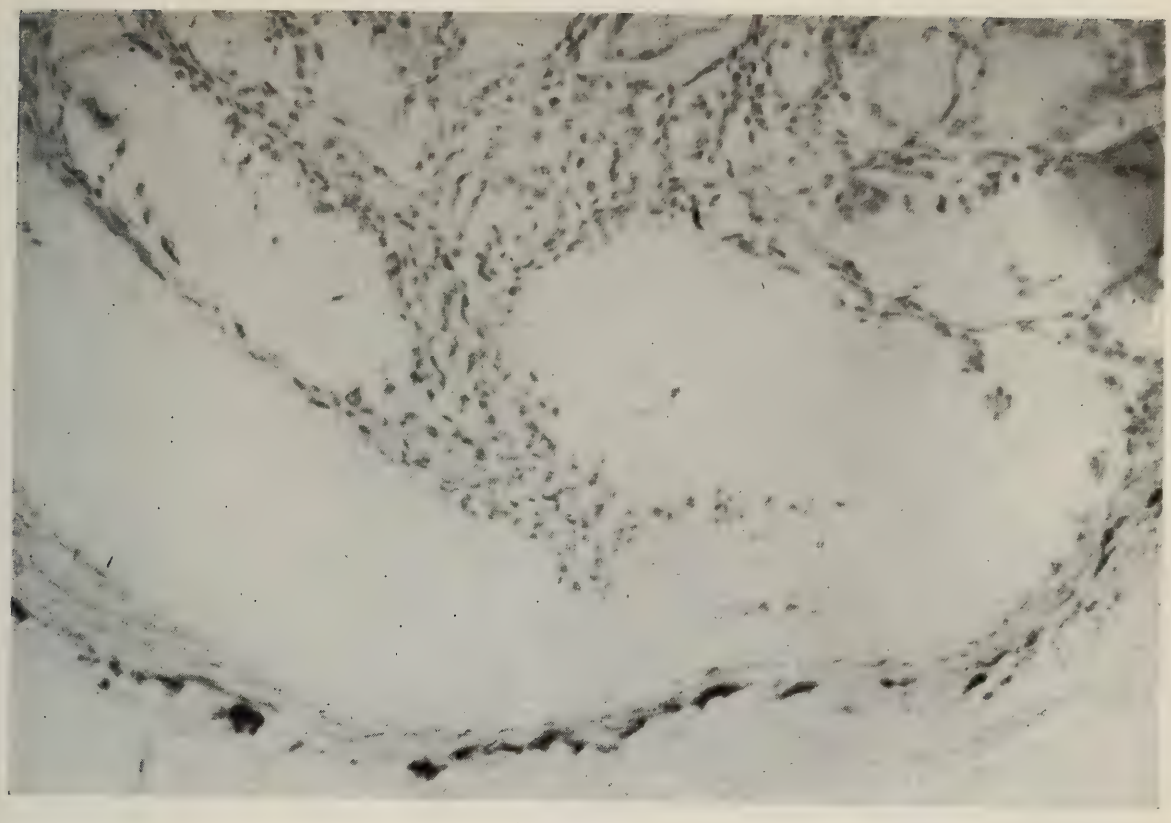


FIG. 13.

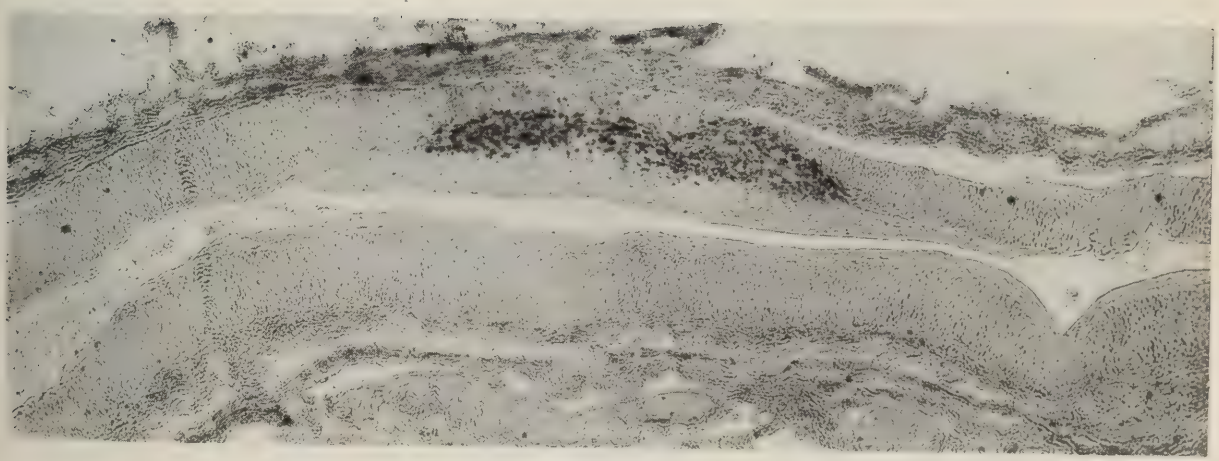


FIG. 14.

(Rous and Oliver: Experimental hemochromatosis.)

[Reprinted from The Journal of Experimental Medicine, November 1, 1918,
Vol. xxviii, No. 5, pp. 645–658.]

URINARY SIDEROSIS.

Hemosiderin Granules in the Urine as an Aid in the Diagnosis of Pernicious Anemia, Hemochromatosis, and Other Diseases Causing Siderosis of the Kidney.

By PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 71 to 76.

(Received for publication, June 17, 1918.)

The siderosis produced in rabbits by transfusions repeated over a long period[1] has as one of its features a pronounced pigmentation of the kidney parenchyma comparable with that encountered in the hemochromatosis of human beings. Great numbers of yellow-brown granules which react positively for iron are present in the cells of the convoluted tubules and the loops of Henle. They are also to be found lying free in the lumen of the tubules, and embedded in casts. This fact suggested a search for hemosiderin granules in the urine of patients with pigmentation of doubtful origin. A test case soon came to hand.

A soldier, aged 46, was admitted to the Hospital of The Rockefeller Institute November 22, 1917. He complained of weakness, loss of weight, sugar in the urine, and swelling of the legs. One brother had died of diabetes at the age of 49. The patient himself had always been strong and well, save for appendicitis relieved in 1900 by operation. A Neisser infection in 1898 was followed by a stricture which yielded to treatment. Lues was denied. The present illness may be said to have begun in 1908, when, following 3 days of hard physical exertion, with loss of sleep, there occurred a sudden edema of the legs. The patient was in bed only a week, and the trouble did not reappear.

In December, 1916, the patient's friends noted that his skin was turning gray. He consulted a physician, who found sugar in the urine. Fasting, followed by a low diet, caused the sugar to disappear; and the patient's weight and strength, which had somewhat diminished, were restored. He entered the army service in

[1] Rous, P., and Oliver, J., *J. Exp. Med.*, 1918, xxviii, 629.

July, 1917, feeling well at that time, but soon began to have swelling of the feet and legs and shortness of breath. The division surgeon found sugar in the urine.

When admitted to the Hospital for treatment by Dr. Allen, the patient was not very ill. He was lean rather than emaciated. The skin of the face and of the back of the hands was somewhat freckled and looked weather-beaten, but more notable was a uniform, light, dusky gray pigmentation of these parts. The remaining body surfaces had no unusual tint, and the mucous membrane of the mouth was normal looking.

Physical examination disclosed a myocarditis, an enlarged, firm liver, ascites, and well marked edema of the feet and legs. The arteries were thickened. The liver edge was 9 cm. below the costal margin in the mm. line, very sharp and firm, and the surface of the organ was smooth, hard, and somewhat bulging. The spleen could not be felt. There was a well marked scar on the glans penis, but the Wassermann reaction was negative. The knee jerks were absent. The urine showed a large amount of sugar, but the ferric chloride test was negative. No albumin or casts were found.

The blood pressure was very low and so remained throughout the several months of hospital observation, ranging between 80 and 104 mm. systolic, and 46 to 70 mm. diastolic. There was a slight anemia: a hemoglobin averaging 85 per cent Sahli, with 4,400,000 red blood corpuscles per c.mm. Reticulated cells were rare on the one examination made, averaging 1 per thousand.

The diagnosis lay between syphilis, favored by the venereal history, genital scar, thickened vessels, absence of the knee jerks, and perhaps also by the enlarged liver, and the diabetes; Addison's disease, as indicated by the pigmentation, low blood pressure, and asthenia; and hemochromatosis, suggested by the combination of enlarged liver, pigmentation, and diabetes. Addison's disease was judged to be most likely.

Urinary Findings.

A fresh specimen of the urine was centrifugated and the slight sediment examined for hemosiderin. It consisted almost entirely of large cells containing numerous coarse, yellow-brown granules (Figs. 1 and 2). Often these granules were as big as red corpuscles, and had then the appearance of having been formed out of several smaller lumps. They were solid looking, moderately refractile, and of irregular shape, in distinction from the rounded, smooth globules of greenish yellow fat, sometimes met with them. Many of the cells were breaking down, and numerous free granules and larger lumps of the pigment were noted. All, both free and intracellular, turned a brilliant black

when submitted to ammonium sulfide, blue with potassium ferrocyanide and hydrochloric acid, and blue too in fixed specimens treated according to Nishimura's method.[2] There could be no doubt that they were composed of hemosiderin. The urine contained no blood, bile, urobilin, albumin, or casts.

The examination of the urine was repeated many times during the next 3 months. The output of hemosiderin granules was regularly abundant, and cells heavily pigmented with them were always obtained in large number from 20 cc. of urine. Control specimens from forty-four patients variously ill were searched for iron by the same methods. With one exception, which may be attributed to artefact, intracellular hemosiderin was found only in diseases involving, like *diabète bronzé*, a true siderosis of the kidney parenchyma. The diseases referred to are pernicious anemia and chronic hemolytic jaundice. In view of these results, the diagnosis of hemochromatosis seemed warranted in the case just described.

Autopsy Findings.

The patient died on April 3, 1918, 3 months after hemosiderin had first been recognized in the urine. Treatment had caused the urinary sugar to disappear promptly and permanently, but hyperglycemia had persisted. There was a gradually increasing edema of the legs and scrotum, with ascites and double hydrothorax; and death ensued from cardiac insufficiency. In the last weeks of life the cutaneous pigmentation became slightly more marked.

An autopsy, $1\frac{1}{2}$ hours after death, performed by the author, disclosed the characteristic lesions of hemochromatosis. The anatomical diagnosis was as follows:

Myocarditis; hemochromatosis with pronounced pigmentation of the liver, pancreas, heart, abdominal lymph nodes, spleen, kidneys, thyroid, mesentery, skin, and other organs; cirrhosis of the liver and pancreas; chronic passive congestion of the liver, spleen, and intestines; ascites; hydrothorax; anasarca; fresh infarct of the lung; old infarcts of the kidneys.

[2] Nishimura, J., *Centr. allg. Path. u. path. Anat.*, 1910, xxi, 10.

The condition of the kidneys has special interest. There were present a few scattered, wedge-shaped areas of local atrophy and connective tissue overgrowth, the result of old infarcts. The general siderosis, though pronounced, was limited to certain groups of cortical elements, notably the distal convoluted tubules (Fig. 3). Here the parenchymal cells were heavily laden with iron-containing pigment, and some had desquamated, while others had broken down *in situ*, liberating pigment granules into the lumen of the tubules (Fig. 4). The cells of the ascending portion of Henle's loop were also pigmented, but less so; and in many of the glomeruli were cells containing numerous fine, hemosiderin granules. The remainder of the kidney was non-pigmented, save for occasional free cells and granules in the lumen of the collecting tubules. The distribution in the organ of the hemosiderin was precisely that which Gaskell and his associates[3] have described and deem characteristic of hemosiderosis.

Method of Search for Hemosiderin.

The following routine method of search for hemosiderin in the urine has proved satisfactory.

A fresh specimen of the urine, preferably warm from the body, is centrifugated at high speed and the supernatant fluid poured off as completely as possible. The sediment is suspended in the trace of fluid that remains, and slide preparations are gone over microscopically for suggestive orange or brown granules, more particularly in the cells. A mechanical stage should be used and 10 minutes at least given to the search. As a rule the sediment from a 20 cc. specimen will yield a fair number of cells from the higher portions of the urinary tract, but often that from 60 to 100 cc. must be obtained. Occasionally, in urine allowed to cool prior to centrifugation, the nubecula is copious and prevents proper concentration of the formed elements. When this is the case, a specimen should be centrifugated as soon as voided. Kept urines which have become cloudy with urates may be cleared by warming. To search a heavy crystalline sediment, or one poor in cells, or containing only leukocytes and squamous epithelium is time wasted. Catheterized specimens are necessary from female patients, since hemosiderin may come into the urine from the genital tract.

The remainder of the sediment is suspended in a fresh mixture of 5 cc. each of 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid. After 10

[3] Gaskell, J. F., Sladden, A. F., Wallis, R. L. M., Vaile, P. T., and Garrod, A. E., *Quart. J. Med.*, 1913–14, vii, 129.

minutes it is thrown down with the centrifuge, the fluid poured off as before, a drop of the sediment mixed on the slide with a drop of the hydrochloric acid solution, and a cover-slip put on. By this modification of Perl's reaction the cells are well preserved and are largely cleared, as are the granular casts. Often the hemosiderin will begin to turn blue as soon as the acid has been added, but, if not, the preparation should be looked at repeatedly in the course of the next half hour. Usually there is present a little foreign debris which turns blue, giving an indication of when a positive finding may be expected. As in the case of the fresh sediment at least 10 minutes of search should be given to the preparation.

The use of a more certain method to demonstrate iron, namely that of Nishimura,[2] is often necessary. It is well known that hemosiderin in the tissues frequently fails to yield Perl's reaction, or to turn black with ammonium sulfide. This is likewise true of urinary hemosiderin. In several pernicious anemia cases suggestive looking orange granules were seen in the fresh, urinary sediment which proved refractory to the ordinary tests but were promptly shown to be iron-containing by the Nishimura procedure. The examination of fresh sediment assumes in this relation considerable importance as indicating that the search for iron should be persevered in. On the other hand, a failure to find suggestive pigment in the fresh specimen does not rule out the presence of iron. The ferrocyanide test has several times disclosed intracellular iron granules so minute or so surrounded by fat globules as not to attract attention in the fresh specimen. By Nishimura's method such instances are found even more often.

For the Nishimura test the fresh sediment, as free as possible from urine, is mixed with a little human serum untinted with hemoglobin and thick films are made and dried. These are fixed by heat, placed in strong ammonium sulfide for 1 hour, washed briefly with water, and subjected for 20 minutes to a fresh mixture of 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid in equal parts. After another brief rinsing with water the preparations are stained in lithium carmine for a few minutes, differentiated in acid alcohol (1 per cent hydrochloric acid), and run rapidly through 95 per cent alcohol, absolute alcohol, xylol, and mounted in balsam. The acid alcohol differentiates the red of the carmine and turns the iron granules a deep blue. Its action should be carefully followed with the microscope, since if prolonged it will dissolve the iron, or at least cause the blue tint of the latter to run and fade. Nishimura carried out two separate differentiations with acid, the first immediately after the ferrocyanide in order to bring out the blue of the iron reaction. But under these circumstances the subsequent action of the acid alcohol tends to destroy the blue color. A single differentiation is more satisfactory.

By this method permanent mounts are obtained to be looked over at leisure. The iron granules stand out in deep blue against the general carmine tint. Less satisfactory preparations may be had by submitting the films to ammonium sulfide and staining thereafter with carmine. Such hemosiderin granules as may react with the sulfide will then appear black. They lose their color rapidly in acid alcohol, so differentiation of the carmine should not be attempted with it.

Specimens left with preservatives prior to examination have proved unsatisfactory. Toluene permits autolysis of the cells, and formalin renders hemosiderin refractory to the ferrocyanide test and at length dissolves it. We have repeatedly found specimens which were negative, preserved with formalin and known to contain iron. Acid also dissolves the pigment, and it need not be looked for in very acid urines save when freshly voided. In old ammoniacal specimens it is remarkably stable and may persist undissolved for several days.

Findings in Pernicious Anemia.

The urines were examined of ten undoubted cases of pernicious anemia. In an eleventh case no proper search for iron could be made because of the heavy sediment of pus corpuscles, etc., incident to a cystitis. In two instances no hemosiderin was found. Repeated search was made of specimens from one of these having the history of a year's illness and a moderate anemia (red blood corpuscles 3,160,000; hemoglobin 69 per cent (Palmer)). In the other case the disease had lasted 3 years, during which period seventeen transfusions had been given. A single specimen only was searched for iron.

Intracellular hemosiderin was found in the urines of all the eight remaining instances, though not always easily. In two a careful search was necessary of specimens stained by the Nishimura method. The cells containing iron were very few and the pigment granules minute (Figs. 5 and 6). In a third instance Perl's reaction, applied to the fresh sediment, disclosed fairly frequent cells containing many fine, blue granules, half obscured amid fat droplets. In the five remaining instances a surprisingly large amount of hemosiderin was found (Figs. 7, 8, and 9). It attracted attention at once in the unstained sediment. Multitudes of fine, brownish, or orange-yellow, granules were seen, grouped in cells, scattered in casts, and more particularly lying free in large numbers. Save in one case, they were much smaller than in the urine of the patient with hemochromatosis already described, as would naturally follow from the relative intensity of the siderosis in the two diseases. From one to fifty or more granules were present in a single cell. They were spherical and remarkably regular in size in different specimens from the same patient, being usually somewhat smaller than eosinophil granulations. Occasionally they had cohered into lumps. The cells containing them were irregularly globular or

cylindrical, slightly larger than leukocytes, as a rule, with a vesicular nucleus of moderate size. Often they were much degenerated and contained myelin or fat. In two cases the modified ferrocyanide test caused the entire sediment to turn bright blue to the naked eye. With the microscope the color was found to be localized in innumerable, sharply defined deep blue granules. In the other instances with abundant hemosiderin the response to the test was only partial, a few of the granules turning blue or green. But with the Nishimura method all of the pigment was shown to be iron-containing.

Hemosiderin was never found in the squamous epithelium or in tailed cells from the bladder. Part, at least, of what was free in the urine obviously came from disintegrating cells. Indeed, the presence in the fresh sediment of small aggregates of orange-yellow granules held together by a remnant of cytoplasm proved significant. In the casts the granules were ordinarily few and widely separated.

A few of the urines showing iron in quantity had the deep, smoky color and the general characters usual in severe pernicious anemia. Others were pale, free from albumin, and seemed normal. No definite relationship could be worked out between the output of pigment granules and the duration and severity of the disease. The fact that pernicious anemia may long exist unsuspected has not improbably much to do with this. The findings in the individual case were remarkably constant from day to day. In one very sick patient, followed through $2\frac{1}{2}$ months, the hemosiderin output remained abundant. This was the exceptional case above mentioned, in which the granules were almost as large and abundant as in the patient with hemochromatosis.

Findings in Hemolytic Jaundice.

Through the courtesy of Dr. George Minot of Boston it was possible to examine the urine of a carefully studied, typical case of hemolytic jaundice of the acquired type. The patient, a male, aged 28, had been more or less jaundiced since the age of 16, following what he understood was an attack of malaria with enlarged spleen. The spleen is still greatly enlarged, but the jaundice is slight, the health excellent, and the blood in good condition. At the time when the urine was searched for iron the blood showed a hemoglobin of 102 per cent Sahli,

but the red cells were rather unequal in size, with many macrocytes and about 6 per cent of reticulated corpuscles. In the urine free hemosiderin was present in quantity, and there were numerous cells stippled with it. Most of the pigment responded readily to Perl's test. The intracellular granules were small, about the size of those usual in pernicious anemia, but much of the free pigment was in coarse, brown lumps. A cast was seen so colored with these as to resemble a miniature cigar.

Findings in Control Cases.

In none of thirty-three control cases, many of them with blood derangements, were such findings as the above obtained. In the fresh urinary sediment the numerous rounded, free, orange or brown granules were lacking, save in one notable instance to be discussed further on, and clumps of them held together by disintegrating cytoplasm were never observed. Pigmented cells, on the other hand, were fairly common. Often they were tinted a diffuse yellow or contained many fine, superimposed, greenish yellow fat droplets. Large yellow or orange-brown globules were also encountered sometimes. In general they were of a fatty or lipoid nature as indicated by their high refractility, smooth outline, resistance to acid, variation in size; and as proved finally by the Scharlach R stain. But occasionally intracellular granules were seen, indistinguishable in the fresh from hemosiderin but negative to microchemical tests. According to Aschoff,[4] an orange pigment, which fails to react for iron, is often present in the kidney cells of old people. A very fine crystalline deposit, furthermore, may simulate hemosiderin granules, free and in casts. Siderosis of the urine cannot be recognized with certainty from the fresh specimen alone. Microchemical tests must always be employed. In this connection it has been interesting to discover that Hunter[5] in 1889 published a note on a granular pigmentation of the cells and casts in the urine of a patient with pernicious anemia. From his description there is no reason to doubt that he had to do with a true siderosis, as was his own conviction. But he made no attempt to demonstrate iron by microchemical tests or to examine the urine of controls. His observa-

[4] Aschoff, L., Pathologische Anatomie, Jena, 2nd edition, 1911, ii, 431.
[5] Hunter, W., *Practitioner*, 1889, xliii, 321.

tion has never been followed up, probably because orange and brown granules are so frequently encountered in urines taken at random.

In one control urine a single cell was found containing iron in the form of several coarse lumps which gave Perl's reaction readily. The urine was an uncatheterized specimen from a woman with a poorly compensated cardiac lesion. No free hemosiderin granules were seen, and a second specimen carefully searched was entirely negative, as was the urine of several other heart cases. In view of the character of the granules and the general circumstances of the case it seems probable that the cell came from the genital tract. The finding emphasizes the necessity for catheterized specimens from female patients.

In another control instance much free hemosiderin was present in the urinary sediment but none in the cells. The patient had a chronic nephritis in the terminal stage with extreme anemia and constant slight bleeding from the mucous surfaces. The urine, of pale straw color, gave a weakly positive guaiac reaction, and a small number of red cells were present in the sediment. This latter contained numerous aggregates of very fine, rounded, slaty brown granules, which reacted positively to the tests for iron. In a second and catheterized specimen obtained 2 days later no red cells or shadows were seen, and there was only the faintest blue coloration on test with guaiac, yet the clusters, or aggregates, of hemosiderin granules were still present. The most careful search failed to reveal any cells containing such granules.

Some of the control cases deserve special mention. Three had the diagnosis of pernicious anemia on admission to hospital treatment, at which time the urine was examined. All had an extremely severe anemia, necessitating transfusion, a high color index, and in two instances a profusion of nucleated red cells were in circulation. One of the cases, that of chronic nephritis, has just been discussed. The other two, with urines negative for both free and intracellular hemosiderin, proved on study to be instances of secondary anemia, one subsequent to a severe purpura, the other associated with chronic malnutrition. The urinary findings were negative also in a patient with chronic polycythemia, and in two instances of Hodgkin's disease with great, progressive anemia.

DISCUSSION.

The presence of hemosiderin granules in the urine seems to have been overlooked heretofore. It is advisable to make a sharp distinction between the intracellular and free pigment, since there is probably a difference in their significance. True, when the granules are found inside urinary cells, free granules are regularly present also, and usually in far greater number. But free hemosiderin has been found alone in the urine of a nephritic patient whose condition presumably did not involve siderosis of the renal cells. Furthermore, extraneous material may easily be mistaken for free hemosiderin by the inexpert. Bits of foreign detritus frequently respond to the test for iron. As a rule, they are coarse and very different in appearance from the finely granular, true pigment.

The free hemosiderin granules occurring in the urine of patients with a true siderosis of the kidney are obviously of intracellular derivation as a rule. The granules have the size and appearance of those in the accompanying cells, and often a remnant of cytoplasm is attached to them or holds together a number of them in its matrix. The rest lie scattered broadcast in the sediment. In one instance, though, the free hemosiderin was of very different character, resembling that in the urine of the nephritic above mentioned. The granules, which were extremely minute, formed aggregates or clusters, sometimes of many hundreds, lying here and there in the urinary sediment (Fig. 10). Intracellular granules were rare. The findings varied little from day to day. The patient, a woman with severe pernicious anemia, had not been transfused, and there was no blood in the urine (guaiac reaction). A possible origin of the pigment from the genital tract was ruled out by catheterization. The inference would seem to be that in this case, as also in the nephritic, hemosiderin was circulating in solution within the body and was directly excreted by the kidney. This assumption is contrary to the general view, according to which during life hemosiderin arises only in cells, and as a granular solid limited to them or their neighborhood.[6] The problem presented is now under investigation in animals.

[6] Brown, W. H., (*J. Exp. Med.*, 1910, xii, 623) has demonstrated that free hemosiderin may be formed in the blood vessels of the autolyzing liver.

The evidence seems convincing that the presence of cells containing hemosiderin in urine obtained under proper precautions is the expression of an actual siderosis of the kidney parenchyma. These cells may be found in most instances, if not all, of diseases that involve such a condition. Fortunately the diseases are few and are readily distinguished from one another, whereas those with which they are oftenest confused produce no renal siderosis. The presence of intracellular hemosiderin in the urine assumes in this light importance for diagnosis.

On *a priori* grounds one may predict with some confidence that the examination of urinary cells for iron pigment will prove to be a decisive method of determining the presence of hemochromatosis in doubtful clinical instances of cutaneous pigmentation. For in hemochromatosis the siderosis of the skin is far surpassed by that of the internal organs. In hemolytic jaundice, congenital or acquired, the history, clinical features, and condition of the blood amply suffice for diagnosis; and the presence or absence of hemosiderin in the urine has interest merely as indicating the condition of the internal organs. In some of these jaundice cases no siderosis of the kidney is found at autopsy.[7] The exact bearing of the urinary findings on the diagnosis of pernicious anemia will require many observations. Positive findings cannot be expected in early stages of the disease before siderosis is established. But do cases come into the physician's hands then? Observations on the urine will throw light upon the point. This much, at least, is already certain, that a positive finding in the urine has significance for the diagnosis of pernicious anemia, whereas a negative one may have none. Even in advanced cases of the disease, as the present work has shown, cells containing hemosiderin may be so rare as to require, by the rather crude methods described, a persevering search for their discovery. Iron pigment is known to be relatively stable, once it has been deposited in the tissues. But whether it will persist sufficiently in the kidney—and the urine—to be useful in diagnosis during the remissions of pernicious anemia remains to be seen.

Besides the diseases mentioned, certain local or general conditions, fortunately rare, may conceivably lead to a urinary siderosis. Severe, long continued hemolysis of any kind, as for example that from malaria,

[7] Guizzetti, P., *Beitr. path. Anat. u. allg. Path.*, 1912, lii, 15.

may be expected to do so. According to Aschoff[4] a patchy siderosis of the renal parenchyma sometimes results from local hemorrhages. Furthermore, according to the same authority, hemoglobin excreted into the tubules may be taken up again by the living cells and converted into hemosiderin. Urinary siderosis becomes a possibility, therefore, in paroxysmal hemoglobinuria, or after the transfusion of incompatible blood. Finally, hemorrhages into the mucosa of the urinary passages may perhaps lead to a desquamation of iron-containing cells.

All but two of the pernicious anemia cases examined in the course of the present work had been transfused, some of them many times. One of the two untransfused patients had much hemosiderin in the urine, and the other none. The influence of successful transfusion to induce of itself a renal siderosis must be considered, since in normal animals transfusions frequently repeated over a long period undoubtedly have this result.[1] But the findings in transfused human beings who were among the controls in the present work are in agreement with the results in animals in showing that much alien blood may be introduced into the body before any renal siderosis develops. The urines of these clinical controls, some of whom had been many times transfused, were uniformly negative for iron-containing pigment granules. In one case, that of a girl of 14 with purpura, nine transfusions, totalling 6,000 cc. of blood, had been given in the month prior to the urinary examinations. Yet the urine, searched on several occasions, proved completely free from hemosiderin. The physiological conditions are different in pernicious anemia. Here transfused blood is in the end just so much more material to be destroyed in the course of the disease, with a resulting increased retention of blood derivatives. That siderosis may be enhanced thereby can scarcely be doubted.

The circulating leukocytes in the case of hemochromatosis were, in general, free from hemosiderin. Several examinations were made, using thick films of washed leukocytes stained for iron by Nishimura's method. After long search one cell was found containing a few small, blue granules.

SUMMARY.

In diseases which bring about a siderosis of the kidney there are ordinarily present in the urinary sediment cells containing granules of hemosiderin, and often many free granules as well. The finding has proved useful in the diagnosis of hemochromatosis and will probably be of service in the recognition of pernicious anemia, and possibly some other diseases. But in this relation the fact should be emphasized that urinary siderosis, as one may term it, is the indication of a renal condition, not of a disease.

My special thanks are due to Dr. Walter W. Palmer, to Dr. George Minot of Boston, and to Dr. Edward Lindeman, for their kindness in providing the clinical material essential to the work.

EXPLANATION OF PLATES.

PLATE 71.

FIG 1. Free and intracellular hemosiderin in the urinary sediment of a patient with hemochromatosis. Nishimura stain.

PLATE 72.

FIG. 2. Drawing of an actual field in a fresh preparation of the same sediment as in Fig. 1. The large size and irregular shape of the pigment granules should be noted.

PLATE 73.

FIGS. 3 and 4. Kidney of the patient with hemochromatosis. Nishimura stain. In Fig. 4 pigmented cells and free granules can be seen lying free in the lumen of a tubule.

PLATE 74.

FIGS. 5 and 6. Minute hemosiderin granules in the urinary cells of a case of pernicious anemia. Nishimura stain. × 1,060.

FIG. 10. Masses of free granular hemosiderin in a urine with scarcely any of the intracellular pigment. The specimen is from a case of pernicious anemia. Nishimura stain. × 1,000.

PLATE 75.

FIGS. 7 and 8. Hemosiderin, free and intracellular, in the sediment from a patient with pernicious anemia. The abundant pigment has the form of rounded granules. One of the cells of Fig. 7 is so crowded with it as to appear almost black. The amount of free pigment is noteworthy.

PLATE 76.

FIG. 9. Drawing to show the appearance of the hemosiderin in fresh preparations from a pernicious anemia case. The cells are taken from several fields.

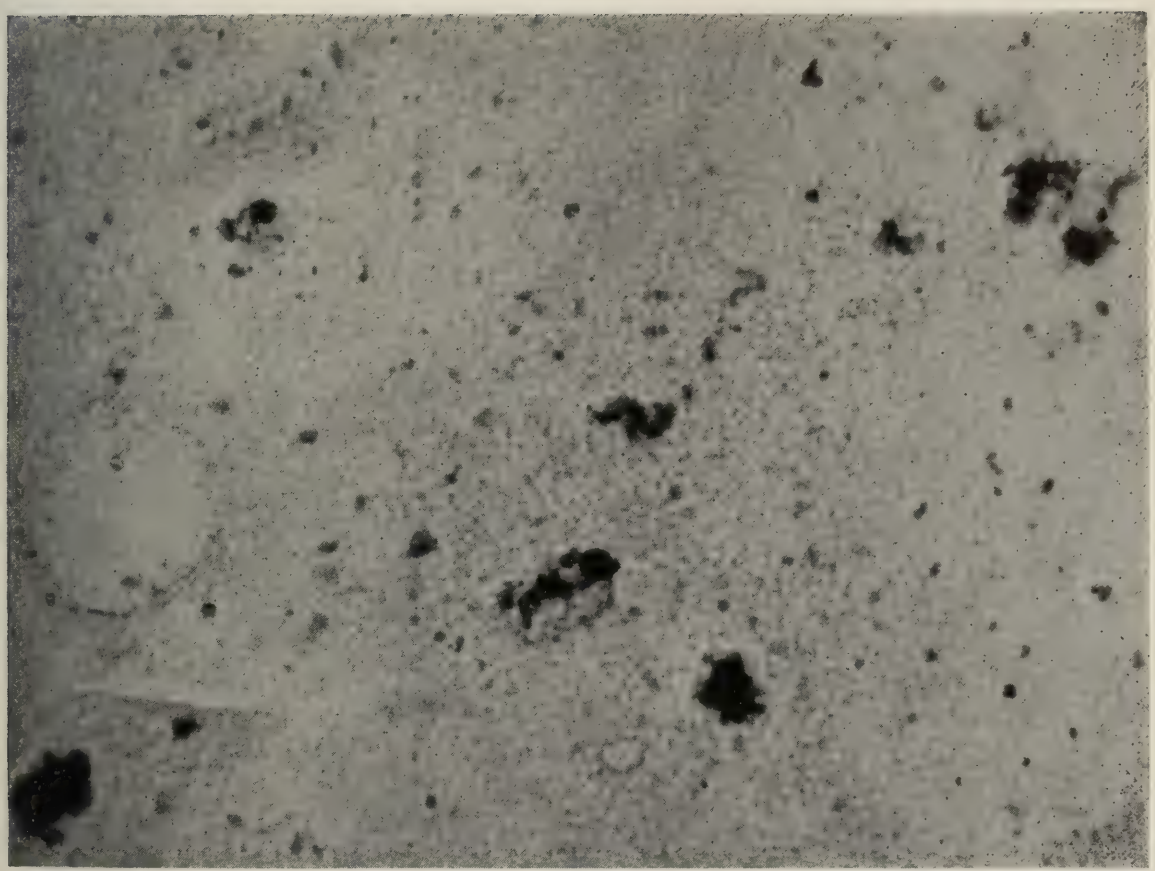


FIG. 1.

FIG. 2.

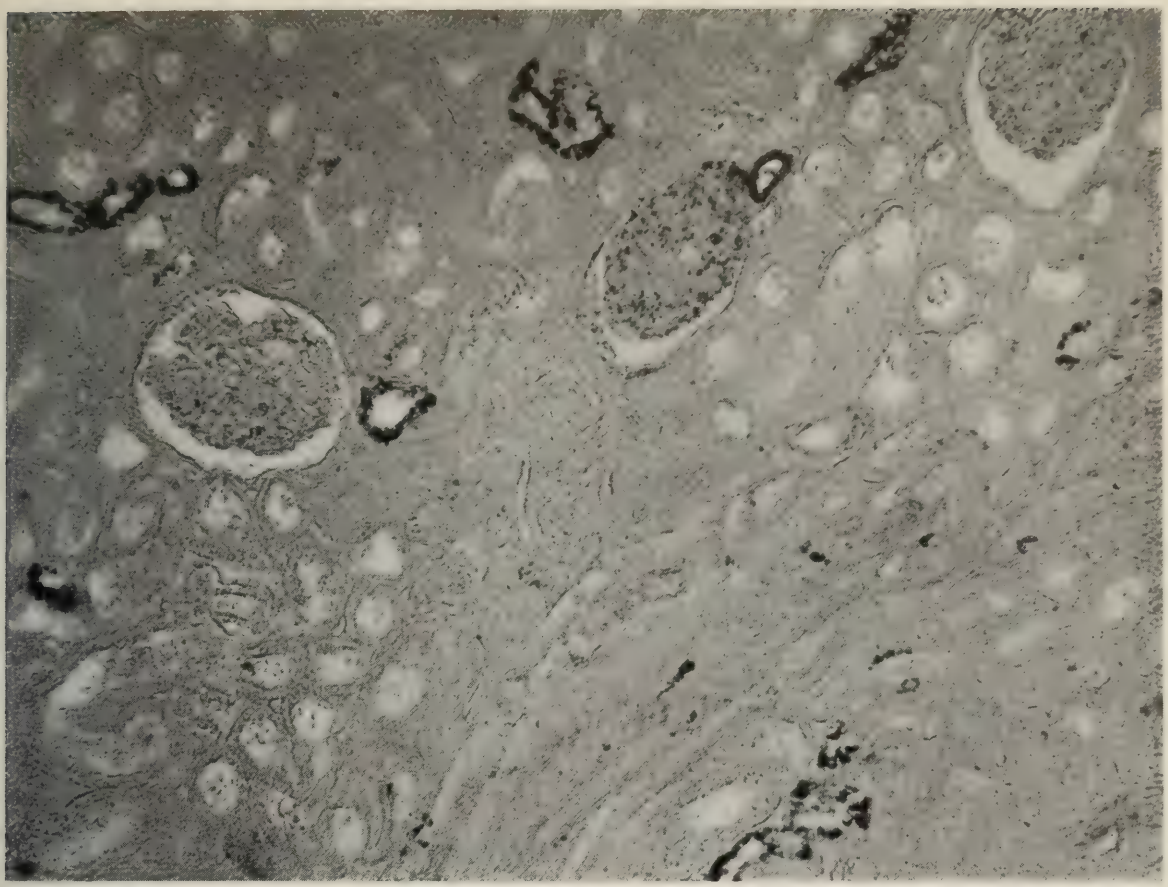


FIG. 3.

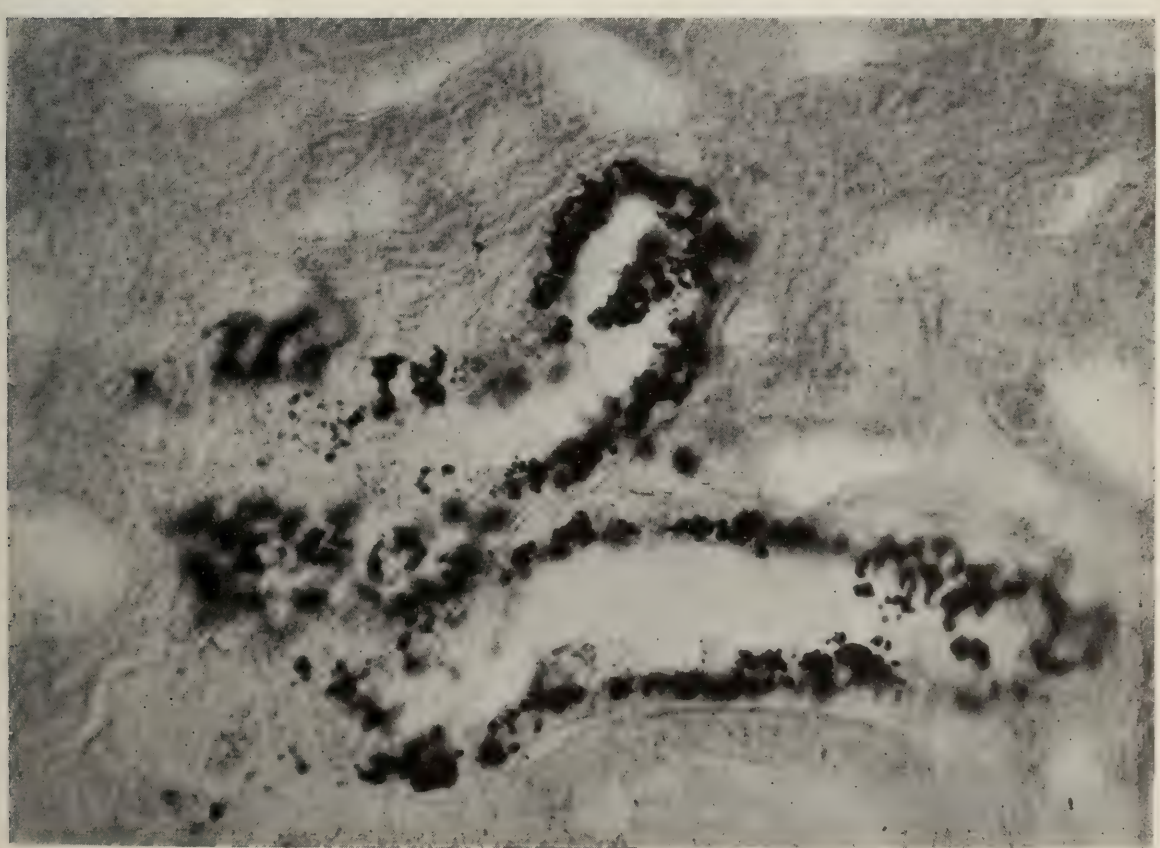


FIG. 4.

(Rous: Urinary siderosis.)

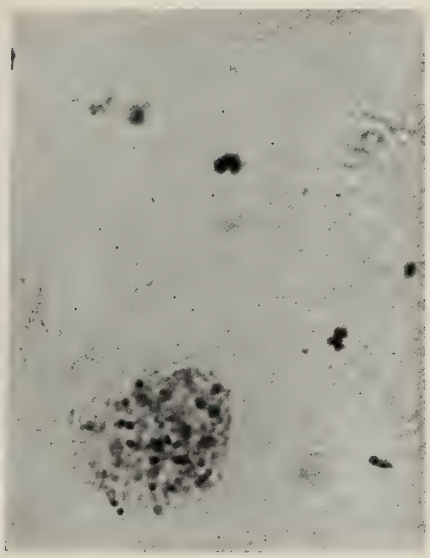


FIG. 5.

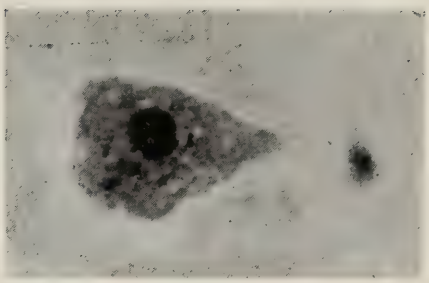


FIG. 6.

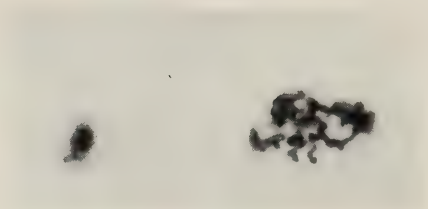


FIG. 10.

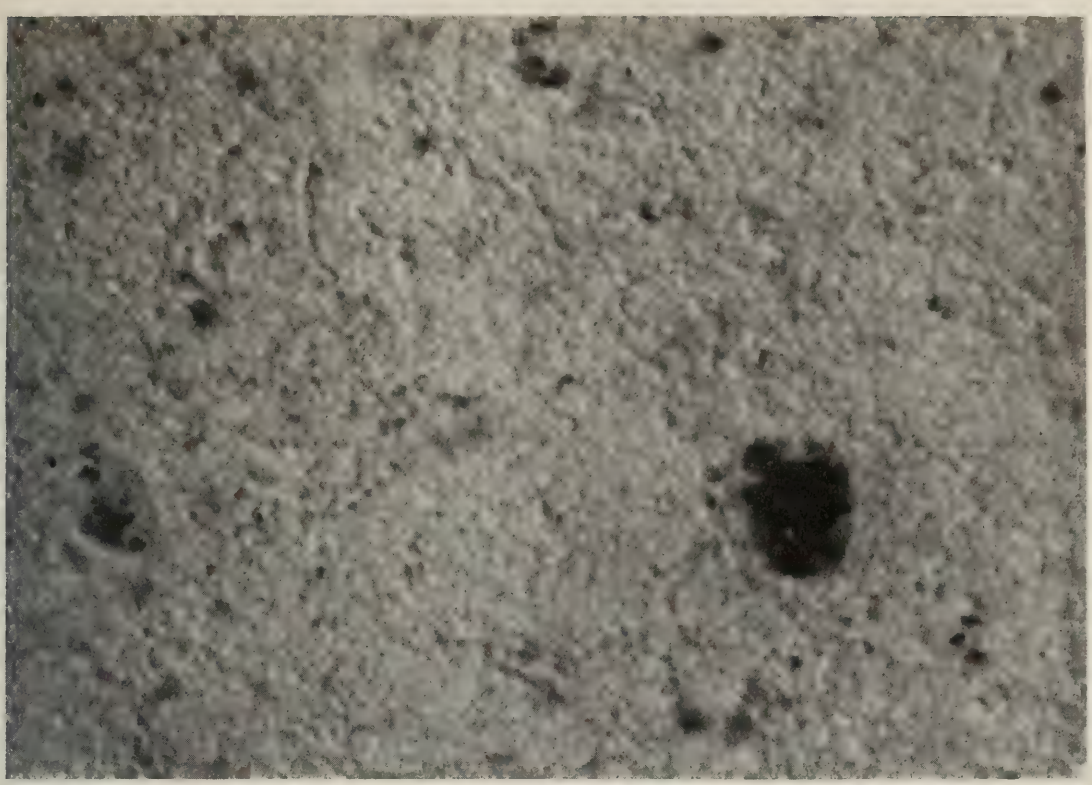


FIG. 7.

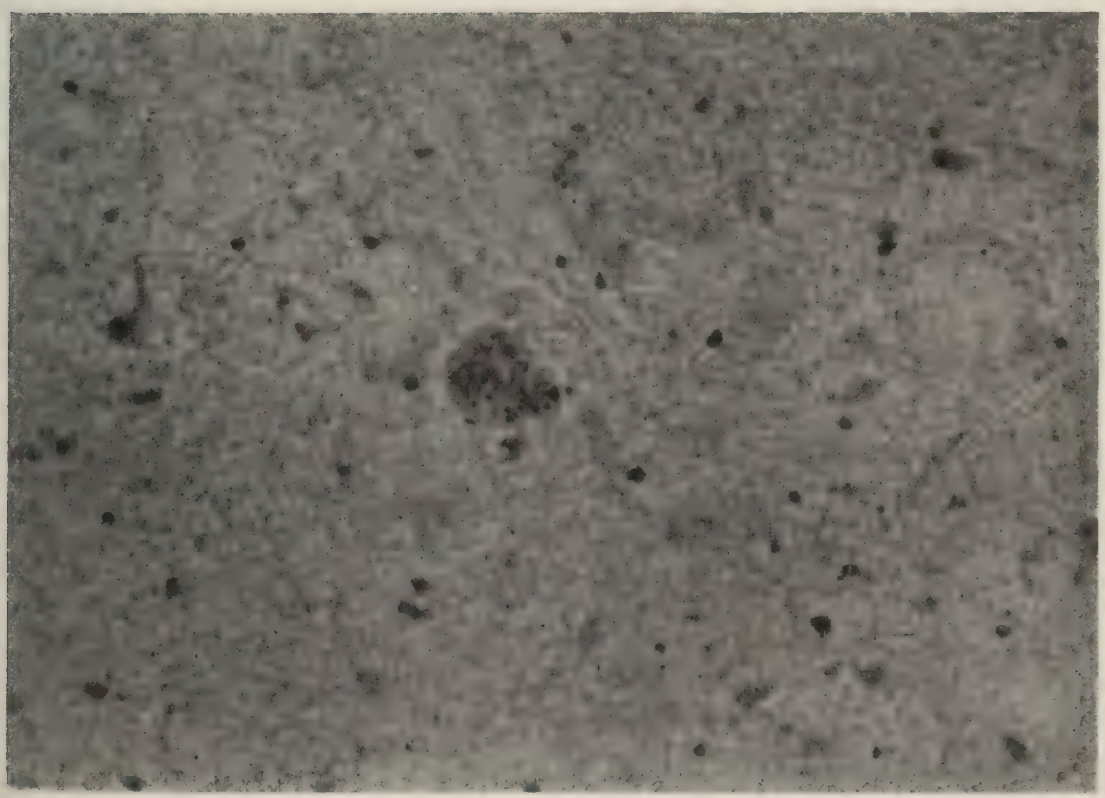


FIG. 8.

(Rous: Urinary siderosis.)

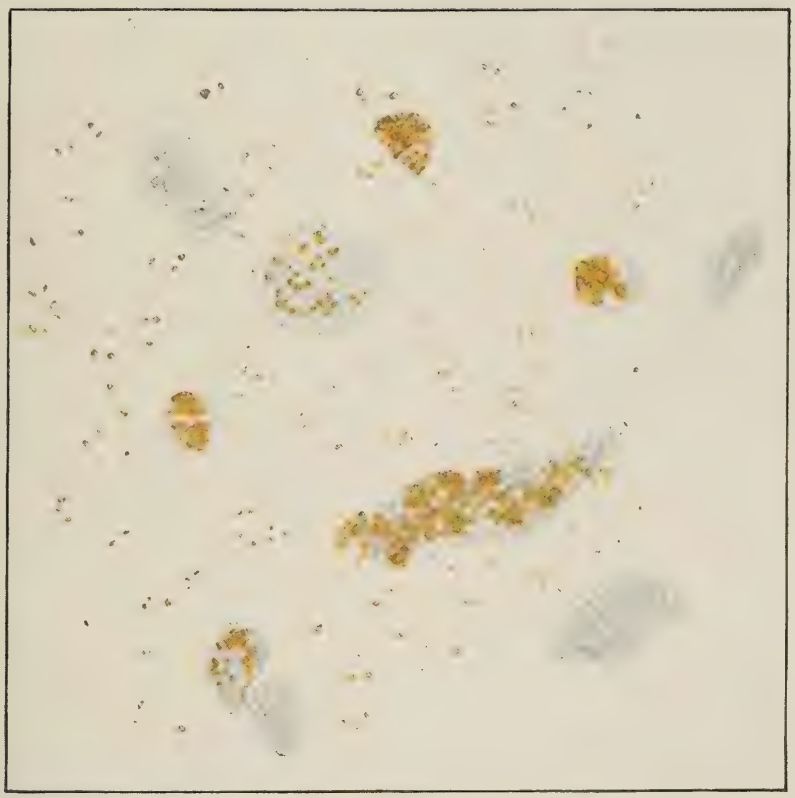


FIG. 9.

[Reprinted from The Journal of Experimental Medicine, December 1, 1918,
Vol. xxviii, No. 6, pp. 681–699.]

RELATIVE IRRITANT PROPERTIES OF THE CHLORINE GROUP OF ANTISEPTICS.

By GLENN E. CULLEN, Ph.D., and HERBERT D. TAYLOR, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 78 to 80.

(Received for publication, September 6, 1918.)

In sterilizing infected wounds with hypochlorite solution one of the most important problems has been that of avoiding excessive irritation. Dakin,[1] found that sodium hypochlorite solution could be used clinically if its concentration was not greater than 0.5 per cent and if the excess alkalinity of the solution had been reduced until there was no longer color with powdered phenolphthalein.

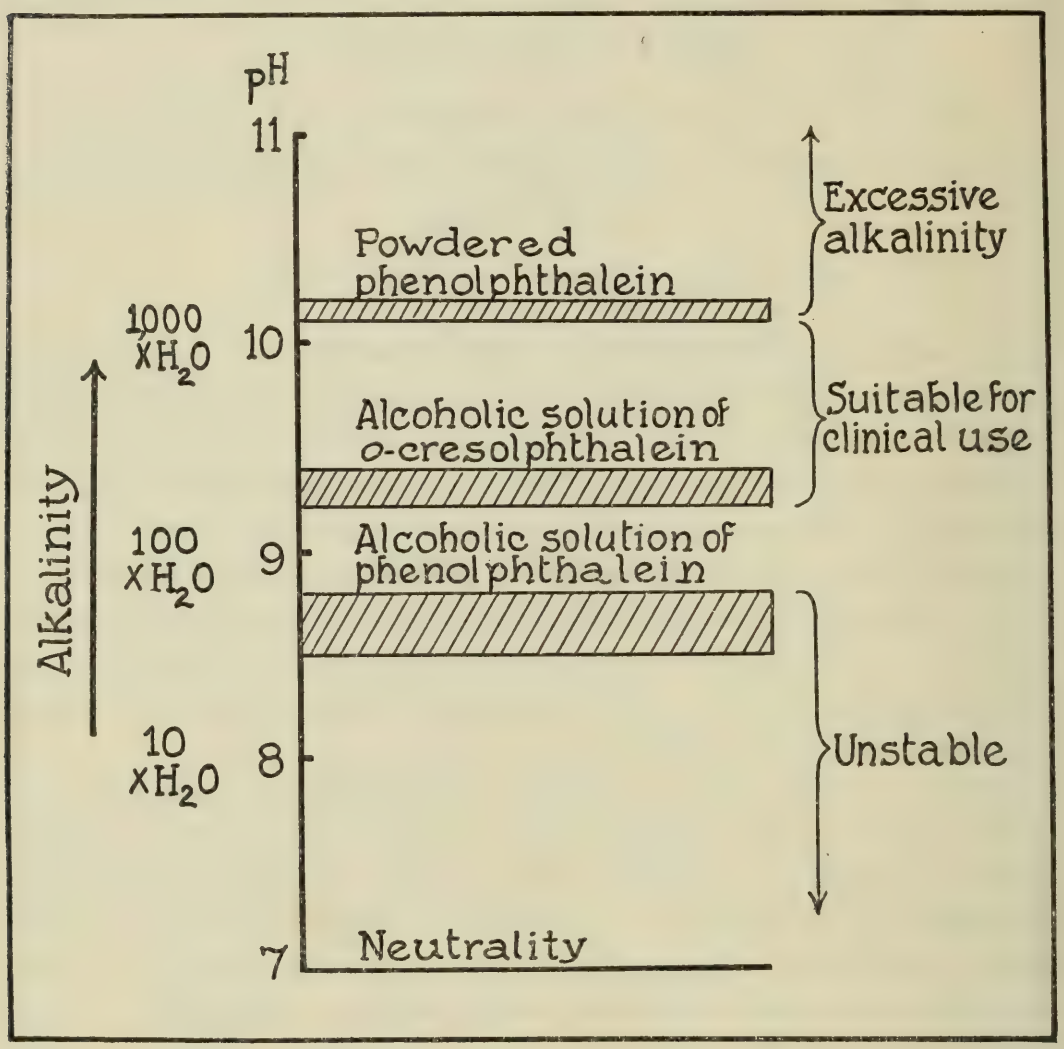
In reducing this excess alkalinity he used boric acid and thus introduced buffer salts that tend to prevent further change in the reaction of the solution. The resulting solution has come into general use as "Dakin's solution."

So many modifications of Dakin's original solution have been proposed that it seemed desirable to gain some idea of their relative irritant properties. Recent work from this laboratory[2] has emphasized the fact that Dakin's hypochlorite solution adjusted to the end-point of powdered phenolphthalein is not a neutral solution as it has often been described, but is a solution with an alkalinity of about 1,000 times that of water (pH = 10.2). Moreover, there is a lower limit of alkalinity, at about 100 times that of water (pH about 9), below which this solution becomes too unstable for clinical use. Powdered phenolphthalein alone is not a sufficient criterion of the reaction, but is the test for the upper limit of alkalinity. The end-points[2] of alcoholic phenolphthalein (pH of 8.5 to 8.8) or *o*-cresolphthalein (pH of 9.2 to 9.4) can be used as tests for the lower limit of alkalinity. It seemed desirable, therefore, in studying the relative irri-

[1] Dakin, H. D., *Brit. Med. J.*, 1915, ii, 318.

[2] Cullen, G. E., and Austin, J. H., *J. Biol. Chem.*, 1918, xxxiv, 553.

tant action of the solutions to be tested, to adjust them to these upper and lower limits of alkalinity. The relation between these limits is expressed graphically in Text-fig. 1.



TEXT-FIG. 1. Approximate alkalinity of a 0.5 per cent sodium hypochlorite solution at end-points to phenolphthalein and *o*-cresolphthalein. Also the relation between alkalinity of solutions and their availability for clinical use. Alkalinity is expressed in terms of hydrogen ion concentration (pH) and compared with that of water.

Three methods are available for the preparation of sodium hypochlorite solutions. In preparing the solution from bleaching powder, the most used and least desirable method, the calcium is precipitated from the bleach suspension by sodium carbonate and the excessive

alkalinity of the filtrate, containing sodium hydroxide (NaOH) and sodium carbonate (Na_2CO_3), is neutralized by boric acid, (Dakin[1]) or by sodium bicarbonate (Daufresne[3]) or by hydrochloric acid and bicarbonate (Cullen and Austin[4]).

The degree of this alkalinity is proportional to the calcium hydroxide content of the bleaching powder which is an extremely variable factor. Neutralization by either boric acid or hydrochloric acid to the end-point to powdered phenolphthalein adjusts the solution to a definite reaction (pH about 10) regardless of this variation. If, however, the same fixed quantity of bicarbonate is added to filtrates of unknown and differing alkalinity, as is the case with the Daufresne modification, the reactions of the resulting hypochlorite solutions will not be constant. Experience has shown us that this modification cannot be used with the bleaching powders of irregular composition obtainable in this country, and we have, therefore, not used it in these experiments.

Sodium hypochlorite solutions may be prepared more conveniently and economically by passing chlorine gas into a sodium carbonate solution.[2] The reaction for a definite hypochlorite concentration is determined by the strength of the carbonate solution used. A third method, that of the electrolysis of a brine solution, is also economical and convenient and is especially suitable for use on ships.[5] Alkali in some form is sometimes added to increase the stability of the solution.

There are many other hypochlorite solutions which have been more or less widely used. A calcium hypochlorite solution and a bleaching powder filtrate, which had been treated with insufficient carbonate to remove all the calcium, were used as types of solutions with low buffer action. Eusol,[6] the solution used extensively by the British Army, is made from equal parts of boric acid and bleaching powder. The boric acid in this case is added to liberate free hypochlorous acid rather than with the purpose of preparing a hypo-

[3] Daufresne, M., *Presse méd.*, 1916, xxiv, 474.

[4] Cullen and Austin, *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 41.

[5] Dakin, H. D., and Dunham, E. K., A handbook of antiseptics, New York, 1918, 116.

[6] Smith, J. L., Drennan, A. M., Rettie, T., and Campbell, W., *Brit. Med. J.*, 1915, ii, 129.

chlorite solution of low alkalinity. The resulting solution is, however, alkaline in reaction. The reasons for the selection of the other solutions will be self-evident.

EXPERIMENTAL.

Choice of Solutions.

We have tested Dakin's solutions made (1) by adjustment of the excessively alkaline bleaching powder-carbonate filtrate with boric acid to the definite end-points of (*a*) powdered phenolphthalein (pH of 10.2) and (*b*) 0.1 per cent alcoholic *o*-cresolphthalein (pH of 9.3); (2) by the adjustment of the same filtrate with hydrochloric acid and sodium bicarbonate to a reaction between these points (Cullen and Austin[4]); (3) by the chlorine-carbonate method to the same reactions (*a* and *b*) as in (1); and (4) by electrolysis. We have further tested (5) a weakly alkaline (nearly neutral) hypochlorite solution; (6) an excessively alkaline hypochlorite solution (dilute Javelle water); (7) sodium hypochlorite solutions containing a small amount of calcium but no carbonate; (8) a calcium hypochlorite solution; (9) a eusol solution; (10) a carbonate control with a pH of 9; and finally the organic chlorine antiseptics introduced by Dakin, (11) chloramine-T[7] and (12) dichloramine-T.[8] For convenience we will refer to the reactions in terms of the pH value. Reference to Text-fig. 1 will show the corresponding alkalinity as compared with water.

Preparation of Solutions.

Solution 1, a (*Bleach-Boric pH of 10.2*).—Dakin's original solution was prepared by mixing 150 gm. of a 25 per cent bleaching powder with a liter of water and allowing this to stand several hours. 100 gm. of sodium carbonate dissolved in another liter of water were added to the bleach suspension. The mixture was shaken vigorously and filtered. The filtrate was neutralized with boric acid until a small sample failed to give color with powdered phenolphthalein, and then diluted with water to a sodium hypochlorite concentration of 0.5 per cent.

---

[7] Dakin, H. D., Cohen, J. B., and Kenyon, J., *Brit. Med. J.*, 1916, i, 160.

[8] Dunham and Dakin, *Brit. Med. J.*, 1917, i, 865. Dakin, H. D., Lee, W. E., Sweet, J. E., Hendrix, B. M., and Le Conte, R. G., *J. Am. Med. Assn.*, 1917, lxix, 27.

Solution 1, b (Bleach-Boric; pH of 9.3).—The solution was prepared exactly as above except that boric acid was added until the solution gave no flash of color with *o*-cresolphthalein. This solution required about five times as much boric acid as did the one at the end-point of powdered phenolphthalein.

Solution 2 (Bleach-HCl-$NaHCO_3$).—The alkaline filtrate obtained as above was neutralized to the end-point to powdered phenolphthalein with 10 per cent hydrochloric acid instead of with boric acid. Then an equal volume of 6.25 per cent sodium bicarbonate solution was added and the solution was diluted with water to 0.5 per cent sodium hypochlorite concentration. This differs from the older use of sodium bicarbonate in that the solution is first adjusted to the definite end-point of powdered phenolphthalein and then made less alkaline by the addition of an arbitrary amount of bicarbonate. The reaction of this solution is slightly more alkaline than the end-point to *o*-cresolphthalein; *i.e.*, is at a pH of about 9.5.

Solution 3, a (Chlorine-Carbonate; pH of 10.2).—The solution was prepared by running chlorine gas into a solution containing 28 gm. of sodium carbonate per liter until it just failed to give color with powdered phenolphthalein. The solution was then diluted with water to 0.5 per cent sodium hypochlorite concentration.

Solution 3, b (Chlorine-Carbonate; pH of 9.3).—The solution was prepared by passing chlorine gas into a solution of 1.4 per cent sodium carbonate to a concentration of 0.5 per cent sodium hypochlorite. This was the routine procedure[9] used in The Rockefeller Institute and this solution was therefore used as a control in later experiments.

Solution 4 (Electrolytic).—The solution was prepared from a 6 per cent sodium chloride solution by the passage of an electric current. The solution gave no color with powdered phenolphthalein, but gave a flash of color with both alcoholic phenolphthalein and *o*-cresolphthalein solution.

Solution 5 (pH Less than 8.5; Nearly Neutral).—The solution was prepared by passing chlorine gas into a carbonate solution until it no longer gave a flash of color with alcoholic phenolphthalein. This solution having a pH of less than 8.5 was then diluted to 0.5 per cent sodium hypochlorite concentration. Since this solution contains a large proportion of hypochlorous acid and is very unstable, it was prepared immediately before each experiment.

Solution 6 (Dilute Javelle Water).—The solution was made from the strongly alkaline filtrate used in preparing the bleach Dakin's by dilution to 0.5 per cent sodium hypochlorite concentration without any neutralization.

Solution 7.—A sodium hypochlorite solution containing no carbonate, but traces of calcium, was prepared by precipitating 95 per cent (determined experimentally) of the calcium of a suspension of bleaching powder with sodium carbonate. The solution was then diluted to 0.5 per cent sodium hypochlorite.

---

[9] A 1.5 per cent sodium carbonate solution is now used.

Solution 8.—Calcium hypochlorite was prepared by filtering a suspension of bleaching powder. The solution was then diluted to a 0.5 per cent sodium hypochlorite equivalent.

Solution 9.—Eusol solution was prepared by mixing 25 gm. of boric acid with 25 gm. of bleaching powder (of about 25 per cent available chlorine) in 1 liter of water. The mixture was allowed to stand over night, filtered, and diluted to 0.5 per cent sodium hypochlorite equivalent. At first there was a definite evolution of gas, but this had ceased before the solution was used. This solution is about twice the concentration of that ordinarily used as eusol.

Solution 10.—An alkaline carbonate control with a pH of about 9 was prepared by adding 1 gm. of sodium carbonate and 13 gm. of sodium bicarbonate to a liter of water.

Solution 11.—Chloramine-T was used in 2 per cent aqueous solution.

Solution 12.—Dichloramine-T was dissolved to a concentration of 5 per cent in chlorcosane.[10]

Testing of Irritation.

The ears of rabbits were used because of the ease with which large surfaces could be exposed to the action of the antiseptic solutions without the irritation of contiguous skin areas. The rabbits were placed on boards with part of the head projecting so that the ears hung over. Two 250 cc. glass tumblers filled with the test solutions were then so placed that each ear was immersed in a separate solution. One ear was placed in the control solution, the other in the solution to be tested.

Single exposures were not sufficient to give definite differences and the experiments were therefore run for either a 20, 40, or 60 minute period per day for several consecutive days. A 60 minute period per day somewhat approximates the length of time that patients under the Carrel[11] method are subjected to the action of hypochlorite solution, for, assuming that the solution maintains effective strength for about 5 minutes at each 2 hour instillation, we would have a total of 60 minutes per day.

Fresh solutions of known concentration were used each day and at the end of each exposure the solutions in the tumblers were

[10] Dakin and Dunham, *Brit. Med. J.*, 1918, i, 51.

[11] Carrel, A., Dakin, Daufresne, Dehelly, and Dumas, *Presse méd.*, 1915, xxiii, 397. Carrel, A., and Dehelly, G., The treatment of infected wounds, New York, 1917.

titrated and the percentage loss of hypochlorite (or hypochlorite equivalent) was recorded. Unless otherwise stated the initial concentration was 0.5 per cent sodium hypochlorite or its equivalent.

The ears were inspected before and after every exposure and daily for 10 days following the last exposure.

Experiment 1.—Nine white rabbits of approximately the same weight were placed in position. The left ear of each was suspended in tap water and the right in the solutions indicated. Each ear was exposed to the same solution 20 minutes daily for 7 successive days. The results are given in Table I.

TABLE I.

Time of Exposure 20 Minutes; Number of Days 7.

Rabbit No.	Right ear.			Left ear.	
	Solution.	Irritation.*	Average fall in concentration.	Solution.	Irritation.
			per cent		
1	0.5% sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+++	11	Water.	0
2	0.5% sodium hypochlorite (bleach-HCl-$NaHCO_3$).	+++	21	"	0
3	0.5% sodium hypochlorite (bleach-boric; pH of 10.2).	++	9	"	0
4	0.5% sodium hypochlorite (chlorine-carbonate; pH less than 8.5).	++++	19	"	0
5	0.5% sodium hypochlorite (with calcium).	+	5	"	0
6	0.5% sodium hypochlorite (strongly alkaline).	++++	11	"	0
7	Alkaline carbonate control (pH of 9).	0		"	0
8	2% chloramine-T.	0	1	"	0
9	5% dichloramine-T in chlorcosane.	++†	0	Chlorcosane.	++†

*In the tables the degree of irritation is indicated as follows:

0, no skin irritation.

+, erosion of hair, slight edema, few petechiæ.

+ +, erosion of hair, considerable edema, more petechiæ.

+ + +, erosion of hair, marked edema, confluent petechiæ.

+ + + +, erosion of hair, very marked edema, confluent petechiæ, and superficial ulceration.

†See discussion.

The solution with a pH of less than 8.5 and the strongly alkaline hypochlorite solutions were the most irritating. The former caused slightly more irritation than the latter. At the end of the experiment the ears were swollen to twice their normal thickness, due to edema, and there were superficial ulceration and confluent petechiæ that discolored the entire surface. The hair had entirely disappeared. The solutions, bleach-boric pH of 10.2, chlorine-carbonate pH of 9.3, and bleach-HCl-$NaHCO_3$, all caused an irritation which was uniformly much less than that of the most irritating group. The bleach-boric pH of 10.2 solution seemed to be slightly less irritating than the others. The ears were edematous, the hair was completely eroded, and subcutaneous petechiæ were rather prominent. The solution containing calcium, but no carbonate, was the least irritating of the hypochlorite solutions. The hair was eroded, there was scarcely any edema, and the few petechiæ present occurred only on the proximal portion of the convexity of the ear. There was no ulceration. The alkaline carbonate control caused no irritation.

Chloramine-T in 2 per cent solution was not irritating. Dichloramine-T, 5 per cent in chlorcosane, produced an effect that differed somewhat from that caused by the other solutions. The congestion was striking; every vessel, large and small alike, seemed to be engorged with blood. There was some thickening of the ear due to a mild grade of edema. There were no hemorrhages and the skin and the hair were not eroded. Chlorcosane alone produced a similar effect but the edema was slightly less marked.

Experiment 2.—This was similar to Experiment 1, save that the exposures were of 40 minutes duration daily for 3 consecutive days. The results (Table II) were identical.

It appeared from Experiments 1 and 2 that the original boric acid Dakin's solution adjusted to an alkalinity of 10.2 was perhaps somewhat less irritating than the solution prepared either from bleaching powder or from chlorine and neutralized to the less alkaline end-point. As this difference was not clear-cut a third experiment was planned to eliminate any individual variations in irritability.

TABLE II.

Time of Exposure 40 Minutes; Number of Days 3.

Rabbit No.	Right ear.			Left ear.	
	Solution.	Irritation.	Average fall in concentration.	Solution.	Irritation.
			per cent		
10	0.5% sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+++	14	Water.	0
11	0.5% sodium hypochlorite (bleach-HCl-$NaHCO_3$).	+++	22	"	0
12	0.5% sodium hypochlorite (bleach-boric; pH of 10.2).	++	27	"	0
13	0.5% sodium hypochlorite (chlorine-carbonate; pH less than 8.5).	++++	30	"	0
14	0.5% sodium hypochlorite (with calcium).	+	11	"	0
15	0.5% sodium hypochlorite (strongly alkaline).	++++	19	"	0
16	Alkaline carbonate (control; pH of 9).	0		"	0
17	2% chloramine-T.	0		"	0
18	5% dichloramine-T in chlorcosane.	++*	0	Chlorcosane.	++*

*See discussion of Experiment 1.

Experiment 3.—The left ears of four rabbits were suspended in bleach-boric solution (pH of 10.2) and the right ears in the routine hospital solution (chlorine-carbonate, pH of 9.3). The ears were exposed 40 minutes daily for 5 consecutive days. They were carefully inspected before and after each exposure to the solutions and daily for 10 days subsequent to the last exposure (Table III).

Rabbit 19 was found dead on the 3rd day. The cause of death was unknown. The skin reactions observed in these rabbits were similar to those observed in Experiments 1 and 2. The hair was eroded from the skin, there were a rather severe grade of edema, congestion, and considerable subcutaneous hemorrhage without, however, any ulceration. At no time during the entire period of observation could any difference be detected between the two ears in any of the rabbits and it was therefore concluded that there was no difference in the irritant actions of these two solutions.

TABLE III.

Time of Exposure 40 Minutes; Number of Days 5.

Rabbit No.	Right ear.			Left ear.		
	Solution.	Irritation.	Average fall in concentration.	Solution.	Irritation.	Average fall in concentration.
			per cent			*per cent*
19*	0.5% sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+	32	0.5% sodium hypochlorite (bleach-boric; pH of 10.2).	+	28
20	" "	++	22	" "	++	15
21	" "	++	26	" "	++	24
22	" "	++	33	" "	++	21

* Found dead on 3rd day.

Experiment 4.—Since there was some variation between individual rabbits in Experiment 3, it seemed desirable to test the other solutions with several animals, using on the control ear a hypochlorite solution rather than water. The solution prepared from chlorine and carbonate at a pH of 9.3 was taken as the control, as in Experiment 3, and the following solutions were tested: the chlorine-carbonate, pH of 10.2, and the bleach-boric, pH of 9.3 (Table IV).

TABLE IV.

Time of Exposure 40 Minutes; Number of Days 5.

Rabbit No.	Right ear.			Left ear.		
	Solution.	Irritation.	Average fall in concentration.	Solution.	Irritation.	Average fall in concentration.
			per cent			*per cent*
23	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3).	++	44	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 10.2).	++	43
24	" "	+++	40	" "	+++	48
25	" "	+++	37	" "	+++	29
26	" "	+++	42	" "	+++	43
27	" "	+++	40	0.5 per cent sodium hypochlorite (bleach-boric; pH of 9.3).	+++	37
28	" "	+++	32	" "	+++	29
29	" "	+++	47	" "	+++	50
30	" "	++	25	" "	++	32

The results show that although individual rabbits may vary slightly in irritability, there is not sufficient difference in the irritant action of the various solutions to be detected by experiments of this nature. The differences are probably not of sufficient consequence to warrant more delicate tests.

Experiment 5.—Results from Experiments 1 and 2 indicate that the sodium hypochlorite solutions containing calcium, but no carbonate or borate buffers, have less irritant action than ordinary buffered solutions. In order to test this with the elimination of the individual variation in irritation shown by different rabbits, four rabbits were subjected to the action of such a solution with the same technique as in Experiments 3 and 4. The results are given in Table V.

TABLE V.

Time of Exposure 40 Minutes; Number of Days 6.

Rabbit No.	Right ear.			Left ear.		
	Solution.	Irritation.	Average fall in concentration.	Solution.	Irritation.	Average fall in concentration.
			per cent			*per cent*
31*	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+++	25	0.5 per cent sodium hypochlorite with calcium.	+++	16
32	" "	+++	23	" "	++	14
33	" "	+++	36	" "	+	21
34	" "	+++	35	" "	+	15

* Died after 5 days.

There is no doubt that these solutions are definitely less irritating than the ordinary Dakin's solutions; however, such solutions should not be substituted for the regular Dakin's solutions without further clinical evidence involving the solvent action on necrotic tissue, pus, effect on rate of cicatrization, etc.

Experiment 6.—The irritant action of electrolytically prepared hypochlorite was compared with that of chlorine-carbonate solution of the same concentration exactly as in Experiments 3 to 5, except that three 60 minute periods were used. As is indicated by Table VI, no difference between the two solutions could be detected.

TABLE VI.

Time of Exposure 60 Minutes; Number of Days 3.

Rabbit No.	Right ear.		Left ear.	
	Solution.	Irritation.	Solution.	Irritation.
35	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+++	0.5 per cent sodium hypochlorite (electrolytic).	++++
36	" "	+++	" "	+++
37	" "	++++	" "	+++
38	" "	++++	" "	++++
39	" "	++++	" "	+++
40	" "	++++	" "	+++
41	" "	+	" "	+
42	" "	++	" "	++

Experiment 7.—The irritant action of eusol, the hypochlorite solution proposed for clinical use by Smith and his colleagues,[6] was compared with that of Dakin's solution in exactly the same manner as in the preceding experiment. It should be noted that the solution is used in a 0.5 per cent sodium hypochlorite concentration rather than about 0.27 as ordinarily used.

TABLE VII.

Time of Exposure 40 Minutes; Number of Days 5.

Rabbit No.	Right ear.			Left ear.		
	Solution.	Irritation.	Average fall in concentration.	Solution.	Irritation.	Average fall in concentration.
			per cent			*per cent*
43	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3).	++	21	0.5 per cent sodium hypochlorite (eusol).	+	17
44	" "	+++	24	" "	++	22
45	" "	+++	19	" "	++	16
46	" "	+++	37	" "	++	24

As will be seen from Table VII, eusol is somewhat less irritating than Dakin's solution. However, in this series of experiments the ears subjected to eusol were affected more for the first 2 days than were those subjected to the Dakin's solution. After 5 days the ears in the Dakin's solution showed slightly more irritation than those in eusol.

DISCUSSION.

The above experiments would indicate that the ears of rabbits are a satisfactory means of studying the irritating effect of solutions on normal skin. In using them, however, to show relative irritation, one should employ an irritating solution as the control, since the rabbits show considerable individual variation in their response to irritation. About one out of each four will develop two or three times more inflammation than the other three. The results may be considered from several view-points, as follows:

Pathology.—Irritation of the ears of the rabbits was evidenced by certain inflammatory changes. Chloramine-T, 2 per cent (Fig. 1), and the alkaline control solution produced no inflammation. No differences could be detected between the ears suspended in these solutions and those in water. Dichloramine-T, 5 per cent in chlorcosane, and chlorcosane alone caused slight edema and marked congestion. The hypochlorite solution containing calcium, but no buffer salts, caused erosion of the hair, slight edema, and a few discrete petechiæ on the proximal portion of the dorsal surface of the ears.

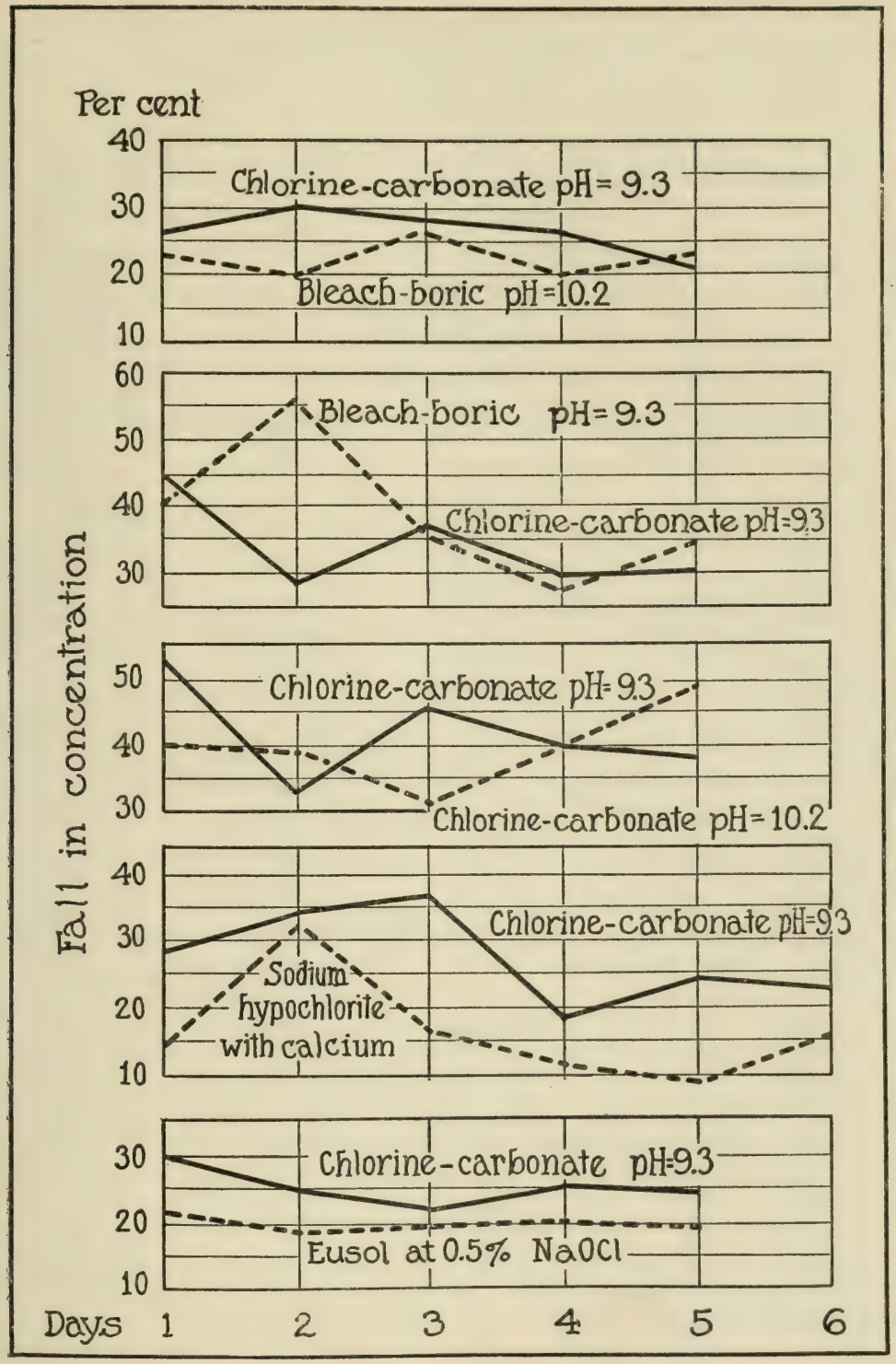
All the Dakin's solutions brought about erosion of the hair, a marked grade of edema, and confluent subcutaneous petechiæ (Fig. 2). The nearly neutral and strongly alkaline hypochlorite solutions eroded the hair, produced a very marked degree of edema, and a discoloration of the entire surface of the skin. There were superficial ulcerations (Fig. 3). Immediately after removal from the hypochlorite solutions the ear surfaces appeared clean and bleached. After a short interval congestion occurred. Between treatments there was a roughing of the surface, probably due to the serofibrinous exudate noted below in connection with the histology. After the first treatment discoloration of the ears occurred, due to petechiæ. After the last exposure a crusting of the exudate took place which fell off after a few days. Gradually the edema disappeared, the subcutaneous hemorrhages were absorbed, and 2 or 3 weeks after treatment new hair began to make its appearance. After another week the ears appeared normal.

Microscopic Examination.—Sections were taken from the ears after the last treatment and stained with methylene blue and eosin, as seen in Figs. 4 and 5.

Chloramine-T produced no demonstrable change (Fig. 4). 5 per cent dichloramine-T in chlorcosane and chlorcosane alone produced very marked congestion and some widening of the tissue spaces (edema). The sodium hypochlorite solution containing calcium also produced a mild degree of edema and brought about erosion of the cornified epithelium and hair and a few subcutaneous hemorrhages. There was an infiltration of small round cells into the subcutaneous tissues and a thin surface exudate, serofibrinous in character. All the Dakin's solutions caused dilatation of the small blood vessels, a rather marked grade of edema, and erosion of the cornified layer of the skin and of the hair shafts which extended down to the hair follicles themselves. There were, in addition, hemorrhages into the subcutaneous tissues, considerable infiltration of the corium with small round cells, and a thickening of the epithelium of the skin and of the hair follicles. Mitotic figures were abundant. There was evidence of fibroblastic proliferation. A thick, shaggy serofibrinous exudate was present over the surface (Fig. 5).

The strongly alkaline and nearly neutral hypochlorite solutions produced changes which were of the same type as those caused by the Dakin's solutions but greater. There were, in addition, superficial ulceration and extensive infiltration of the subcutaneous tissues with polymorphonuclear leucocytes.

Rate of Decomposition of Solutions.—It is, of course, well recognized that the chlorine antiseptics are decomposed by tissue material. To determine whether there was any relation between the extent of irritation and the rate of deterioration of the antiseptic, the solutions in Experiments 3, 4, 5, and 7 were titrated after each experiment. The results were analyzed in several ways. First, the percentage fall in concentration per day for each solution was studied and plotted as curves. There were so many irregularities in these curves that the composite averages for each set of four ears were compared. The composite daily curve of the four ears in each set subjected to the chlorine-carbonate control solution may be compared with the composites of the four ears in the other solutions. The curve plotted from such averages for Experiments 3, 4, 5, and 7 are given in Text-fig. 2. It would seem that while the irregularities in the curves are marked, there is a definite tendency for the solutions that were the



TEXT-FIG. 2. Curves showing fall in concentration of hypochlorite solutions in contact with ears of rabbits. Each curve represents the composite of the results obtained with the four rabbits used in each experiment. The fall in concentration is expressed as per cent of the initial concentration.

least irritating, to show a lower percentage loss. This is, of course, to be expected, for the greater the irritant action, the more marked are

TABLE VIII.

All Solutions 0.5 Per Cent Sodium Hypochlorite or Its Equivalent.

Experiment No.	Rabbit No.	Right ear. Solution.	Right ear. Period average fall for each animal.	Right ear. Period average fall for set of animals.	Left ear. Solution.	Left ear. Period average fall for each animal.	Left ear. Period average fall for set of animals.
			per cent	*per cent*		*per cent*	*per cent*
3	19	Chlorine-carbonate; pH of 9.3.	32		Bleach-boric; pH of 10.2.	28	
3	20	" "	22		" "	15	
3	21	" "	26		" "	24	
3	22	" "	33	28	" "	21	22
4	23	Chlorine-carbonate; pH of 9.3.	44		Chlorine-carbonate; pH of 10.2.	43	
4	24	" "	40		" "	48	
4	25	" "	37		" "	29	
4	26	" "	42	41	" "	43	41
4	27	Chlorine-carbonate; pH of 9.3.	40		Bleach-boric; pH of 9.3.	37	
4	28	" "	32		" "	29	
4	29	" "	47		" "	50	
4	30	" "	25	36	" "	32	37
5	31	Chlorine-carbonate; pH of 9.3.	25		Sodium hypochlorite and calcium.	16	
5	32	" "	23		" "	14	
5	33	" "	36		" "	21	
5	34	" "	35	30	" "	15	17
7	43	Chlorine-carbonate; pH of 9.3.	21		Eusol.	17	
7	44	" "	24		"	22	
7	45	" "	19		"	16	
7	46	" "	37	25	"	24	20
Average for 20 rabbits.....				32			28

the roughening of the skin surface and consequent increase in area. The serous exudate and bits of tissue from the irritated surfaces would also react with and decompose the solutions.

Secondly, the results for each ear over the entire period were then compared. The average percentage loss over the entire period, *i.e.* 3 to 6 days for each ear, are given with each experiment as the "period average fall" and summarized in Table VIII. It will be seen that the average fall of the solution used most, chlorine-carbonate (pH of 9.3), for the entire twenty ears is 32 per cent and that the average fall for the twelve ears subjected to the other Dakin's solutions is 33 per cent. It should be recognized that changes in temperature, season, etc., may cause considerable variation on different days. It is, however, striking that in the solutions which cause similar biological changes, the average losses are almost identical.

CONCLUSIONS.

From the above observations we must conclude that Dakin's hypochlorite solutions, of which the alkalinity is kept within the range of from 100 to 1,000 times the alkalinity of water (pH of 9.3 to 10.2) by means of buffer salts, have practically the same degree of irritant action. Moreover, the manner in which these solutions are prepared, whether from bleaching powder and sodium carbonate, or from chlorine and sodium carbonate, and the type of buffer salt used, either carbonate or borate, have no influence on the degree of irritation. Solutions, however, that have an alkalinity less than that indicated by the end-point of alcoholic solution of phenolphthalein (pH of 8.5 to 8.8), or greater than that indicated by the end-point to powdered phenolphthalein (pH of 10.2), are intensely irritating. The sodium hypochlorite solution from which most of the calcium has been precipitated, and calcium hypochlorite solution seem less irritating than Dakin's solutions. The alkalinity in these unbuffered solutions is due mainly to the hydrolysis of the hypochlorite and is decreased as the hypochlorite is decomposed. This, rather than any specific action of the calcium, is probably the explanation of their lessened irritant action.

It is also evident that one may expect the hypochlorite solutions to maintain effective antiseptic concentration for about the same period of time, regardless of the manner in which they are prepared.

SUMMARY.

1. The use of the ears of rabbits is proposed in testing the irritant effect of antiseptics.

2. It is necessary, because of individual variations, to use solutions having definite irritant actions as controls.

3. 0.5 per cent sodium hypochlorite solutions have minimum irritant effects over a range of alkalinity of from about 100 to 1,000 times that of water (pH about 9 to 10). Solutions may be adjusted within these limits by use of the end-points of powdered phenolphthalein, of alcoholic solutions of either *o*-cresolphthalein or phenolphthalein.

4. Sodium hypochlorite solutions kept within the above range of alkalinity by either borate or carbonate buffer salts, *i.e.* Dakin's solution, show the same irritative properties whether made from bleaching powder and sodium carbonate, or from chlorine and sodium carbonate.

5. Electrolytically prepared solutions of the same concentration have similar irritant action.

6. Solutions, however, that have an alkalinity less than that indicated by the end-point of alcoholic phenolphthalein solutions (pH of 8.5 to 8.8) or greater than that indicated by the end-point to powdered phenolphthalein (pH of 10.2) are intensely irritating.

7. 0.5 per cent sodium hypochlorite solution from which most of the calcium has been precipitated and calcium hypochlorite solution of equivalent hypochlorite concentration are only slightly irritating.

8. 2 per cent chloramine-T solution has no irritant action.

9. 5 per cent dichloramine-T in chlorcosane and chlorcosane alone irritate rabbit ears to a slight degree only.

EXPLANATION OF PLATES.

PLATE 78.

FIG. 1. Rabbit 8, Experiment 1. Right ear suspended 20 minutes daily for 7 days in 2 per cent chloramine-T solution. Left ear suspended for same time in water.

FIG. 2. Rabbit 2, Experiment 1. Right ear suspended 20 minutes daily for 7 days in 0.5 per cent sodium hypochlorite (bleach-HCl-$NaHCO_3$). Left ear suspended for same time in water.

PLATE 79.

FIG. 3. Rabbit 4, Experiment 1. Right ear suspended 20 minutes daily for 7 days in 0.5 per cent sodium hypochlorite (chlorine-carbonate; pH less than 8.5). This figure shows the effect of hypochlorous acid. Left ear suspended for same time in water.

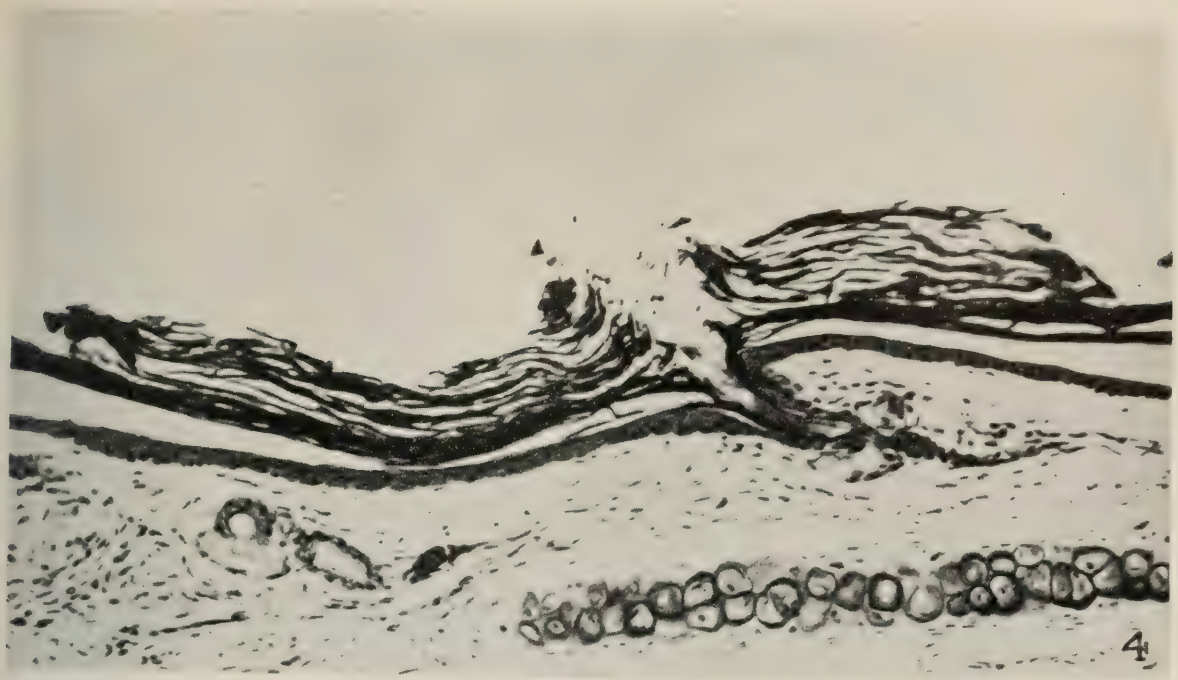
PLATE 80.

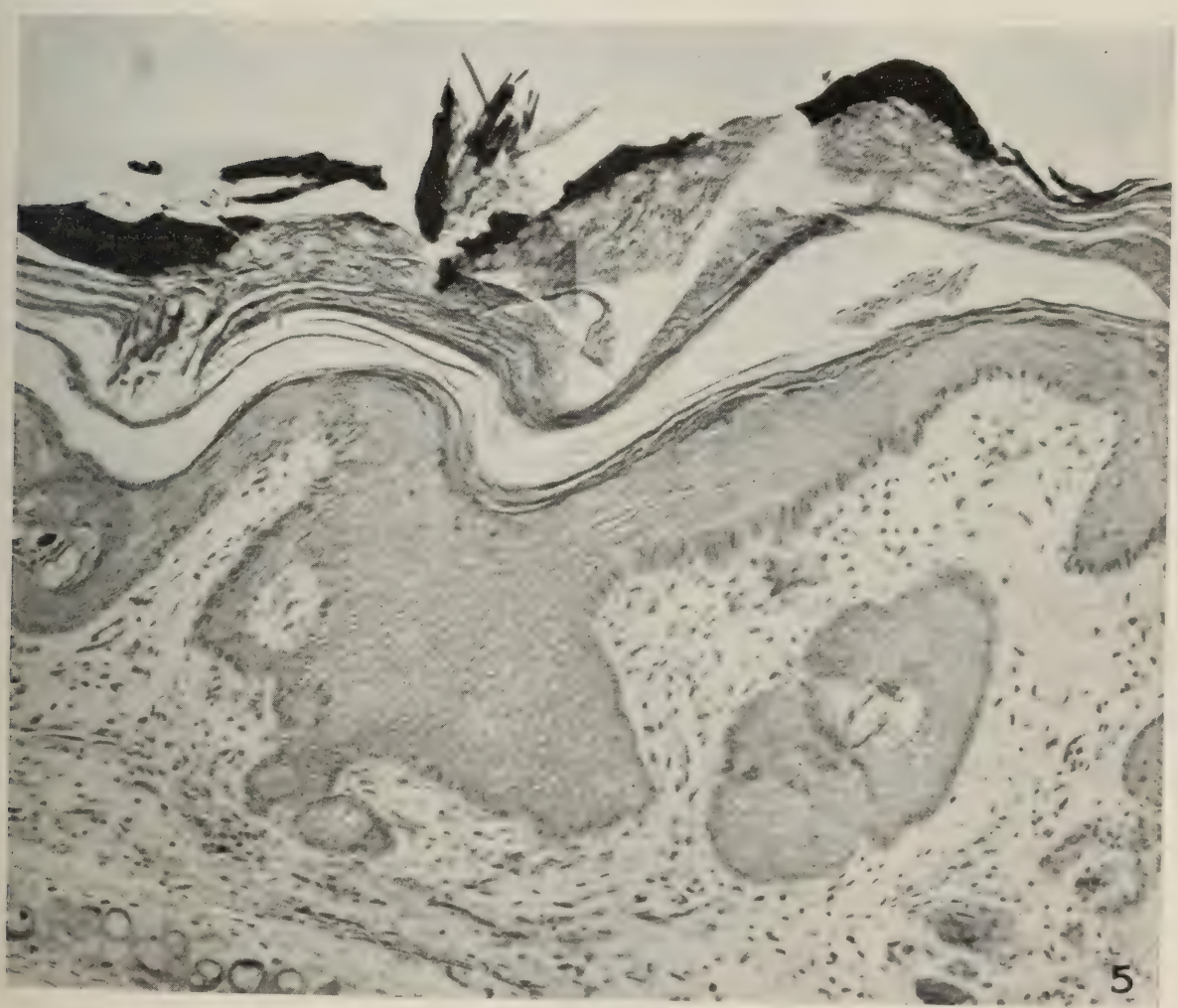
FIG. 4. Section of the skin of the ear of Rabbit 8, Experiment 1, treated for 20 minutes daily for 7 days with 2 per cent chloramine-T solution. × 140.

FIG. 5. Section of the skin of the ear of Rabbit 2, Experiment 1 (Fig. 2), treated for 20 minutes daily for 7 days with 0.5 per cent sodium hypochlorite (bleach-HCl-$NaHCO_3$). × 140.

(Cullen and Taylor: Irritant properties of chlorine antiseptics.)

(Cullen and Taylor: Irritant properties of chlorine antiseptics.)





(Cullen and Taylor: Irritant properties of chlorine antiseptics.)

[Reprinted from The Journal of Experimental Medicine, December 1, 1918,
Vol. xxviii, No. 6, pp. 779–790.]

STANDARDIZATION OF ANTIMENINGOCOCCIC SERUM.

By HAROLD L. AMOSS, M.D., and PENELOPE MARSH.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 17, 1918.)

The establishment of antimeningococcic serum as a specific therapeutic agent for epidemic or meningococcus meningitis antedated Dopter's[1] discovery of the duality of meningococci. Previous to Dopter's studies the effort to produce a so called polyvalent antimeningococcic serum was wholly empirical. Several or many different strains of meningococcus were employed for inoculation on the supposition that such biological variations as occurred in: he species would be thus covered. The discovery by Dopter of the parameningococcus necessitated a change of procedure. The strains used for inoculation of horses were now chosen according to whether they conformed serologically to the so called normal or regular meningococcus or the parameningococcus. Wollstein,[2] who studied two of Dopter's cultures as well as a considerable number of strains isolated in the United States, confirmed not only the serological distinctions between normal and parameningococci, but made the important observation that between these two extreme types a number of intermediate varieties were intercalated. These intermediates inclined either toward the normal or the para organisms but were distinguishable serologically from both.

The studies of the meningococcus stimulated by the appearance of epidemic meningitis in the armies of all the belligerent powers have served to confirm and in some degree to extend the observations of Dopter and of Wollstein. The present point of view is represented

[1] Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxvii, 74.

[2] Wollstein, M., *J. Exp. Med.*, 1914, xx, 201.

by the classifications of Gordon[3] and of Nicolle and his associates.[4] Briefly stated they distinguish also two main types of meningococci designated either Types I and II or A and B. Gordon also recognizes Subtypes III and IV, one affiliating with Type II and the other with Type I, and Nicolle equally distinguishes two such types, C and D, which show similar affiliations.

The serological subdivision of the meningococcus into varieties or types has undoubtedly marked a forward step in our knowledge of the causation and specific treatment of epidemic meningitis. It may, however, perhaps be regarded as of questionable value to set up too many minor varieties or types. The conditions are not dissimilar to those existing among the bacilli of dysentery with which we have long been familiar. In both instances the bacterial groups—meningococci and dysentery bacilli—seem still to be in a state of specific flux. In the case of meningococcus this fact is made especially apparent by the study of carrier strains which show even greater serological fluctuation than do the strains obtained from the inflamed meninges.[5]

However, the present chief interest centers around the establishment of a test of therapeutic efficiency in antimeningococcic serum. It does not yet appear feasible to treat epidemic meningitis on the basis of monovalent sera adapted in every instance to the particular type or variety of meningococcus inducing the infection. Nor does it seem necessary to attempt this. There are no insuperable difficulties in the way of preparing an adequate polyvalent antimeningococcic serum in which not only the two main kinds of antibodies are sufficiently represented but also the main subvarieties or types as well. Meningococci do not exhibit such strict serological specificity as we have become familiar with in regard to the types of pneumococci.[6] On the contrary, there is much overlapping of antibody

[3] Gordon, M. H., *Great Britain National Health Insurance, Med. Research Com., Special Rep. Series, No. 3*, 1917, 10.

[4] Nicolle, M., Debains, E., and Jouan, C., *Ann. Inst. Pasteur*, 1918, xxxii, 150.

[5] Eastwood, A., *Rep. Local Gov. Bd. Pub. Health and Med. Subjects*, 1917, N.S. cxiv, 1. Griffith, F., *ibid.*, 52. Scott, W. M., *ibid.*, 111. (See also *J. Hyg.*, 1918, xvii, 63, 124, 191.) Gordon, M. H., *J. Hyg.*, 1918, xvii, 290.

[6] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

reactions, a fact which affects undoubtedly the wide therapeutic applicability of the antimeningococcic serum.

In spite of this overlapping it is imperative that a standard should be established which will afford a measure of the therapeutic efficacy of given samples of the serum. An efficient sample of the serum should contain definite amounts of antibodies for the principal varieties and the main subvarieties or types of the meningococcus. There will be no disagreement as to the two main types, while differences of opinion may arise as to the main subvarieties. As regards the latter it will be safe to follow Gordon's classification which appears to express the essential fact. Accordingly the polyvalent antimeningococcic serum should be prepared with at least four properly chosen cultures of the meningococcus. This is the point of view arrived at by the New York State Department of Health, which has adopted and is enforcing in the State a standard based on agglutination titer.[7]

In choosing a standard of value for the antimeningococcic serum certain questions at once arise. The first relates to the particular antibody on which therapeutic activity depends. The point at issue with an antibacterial serum, as represented by the antimeningococcic serum, is far more complex than with the ordinary antitoxic sera. According to present beliefs the antimeningococcic serum acts (*a*) by increasing phagocytosis of the meningococcus,[8] (*b*) by neutralizing endotoxin,[9] (*c*) by injuring directly the meningococci and impairing their power of propagation.[10] It remains, therefore, to be determined whether any one criterion will suffice as a measure of these several forms of activity.

The methods employed to effect the standardization of the serum have from time to time undergone change. Thus there have been employed successively (*a*) opsonin content,[11] (*b*) complement-fixing

[7] *Off. Bull., New York State Dept. Health*, 1918, iii, No. 5.

[8] Jochmann, *Deutsch. med. Woch.*, 1911, xxxvii, 1733. Flexner, S., *J. Exp. Med.*, 1907, ix, 168. Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1916, xxiii, 683.

[9] Flexner, S., *J. Exp. Med.*, 1907, ix, 168.

[10] Flexner, *J. Exp. Med.*, 1907, ix, 105. Flexner, S., and Jobling, J. W., *J. Exp. Med.*, 1908, x, 141.

[11] Jobling, J. W., *J. Exp. Med.*, 1909, xi, 614.

titer,[12] (*c*) agglutinin content,[13] (*d*) antiendotoxic value,[14] and (*e*) protective power.[15]

The discrimination of parameningococcus from the regular meningococcus has automatically rendered the opsonic and complement-binding methods obsolete, since they do not suffice for the separate determination of the discrete antibodies. In practice, the discussion of standards has narrowed itself down to the relative merits of agglutinins or protective power as a measure of value.

It is desirable to state here that agglutination is the method of choice for standardization, since it alone gives clear distinctions between the types and varieties of meningococci. Its employment has been questioned on the ground that as the therapeutic action of the serum is probably not a function of the agglutinin, antibody may not be a measure of therapeutic efficacy. An effort is being made, therefore, to substitute for the agglutinin content of the serum the protective power.

In endeavoring to set up a standard of protection, account must, in the first instance, be taken of the small and variable pathogenic action of the meningococci. This fact has indeed been generally recognized by investigators. While certain small animals, such as young guinea pigs weighing about 100 gm.[16] and mice, will often succumb to intraperitoneal inoculations of moderate doses of cultures of meningococci, yet their reaction is variable, and fluctuation in virulence of the cultures is common.

EXPERIMENTAL.

Recently, therefore, Hitchens and Robinson[15] have proposed a method of standardization of the antimeningococcic serum based on its protective power. Because of the intrinsic importance of the subject for our military organizations and our civil communities, we have submitted the method to a critical examination.

---

[12] Krumbein and Schatiloff, P., *Deutsch. med. Woch.*, 1908, xxiv, 1002.

[13] Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403. Amoss, H. L., *J. Am. Med. Assn.*, 1917, lxix, 1137.

[14] Flexner, *J. Exp. Med.*, 1907, ix, 168. Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxvi, 772. Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1908, xxi, 12. Gordon, M. H., *Brit. Med. J.*, 1918, i, 110.

[15] Hitchens, A. P., and Robinson, G. H., *J. Immunol.*, 1916, i, 345.

[16] Flexner, *J. Exp. Med.*, 1907, ix, 168.

Hitchens and Robinson give in their paper what we regard as illustrative protocols of their experiments, from which they conclude that the "protection test parallels the extent of immunization more nearly than agglutination or complement fixation tests." They also state that "there seems to be a considerable degree of specificity in the test." A close study of their protocols fails, we believe, to carry conviction that, even in their hands, the test is either delicate or decisive. Unless it is both delicate and decisive, the object which they seek, namely that "if the amount of serum necessary to protect against one M. L. D. of culture is considered as a unit, a rational and uniform method of standardizing antimeningococcus serum can be obtained," and "a dosage similar to that of antitoxic serum would be possible by such a method of standardization" is not attained.

In carrying out our experiments we have followed closely the conditions laid down by Hitchens and Robinson. Thus a 16 hour growth of the meningococcus chosen was suspended in 1 cc. of dilute guinea pig serum and injected immediately in the indicated doses into the peritoneal cavity of white mice. In testing the action of samples of the antimeningococcic serum, 0.5 cc. was injected intraperitoneally 2 hours before the suspension of the culture was inoculated also intraperitoneally. Finally, in instances in which several cultures of a given meningococcus were made, they were all washed off with the dilute guinea pig serum and pooled before being used for inoculation. The volumes of serum and of bacterial suspension employed have always been 0.5 cc. each.

Series I. Regular Meningococcus.

A regular meningococcus having a suitable degree of virulence was selected. The suspension was prepared in the manner indicated and injected immediately into the peritoneal cavity (Table I). As Table I shows, all but one of the injected mice died; but the order of their death did not conform to the dosage of the culture. When death took place within 24 hours the peritoneal cavity and heart's blood yielded many or innumerable colonies of meningococcus; when death was delayed the number of colonies grown from the heart's blood was often few.

The next experiment was made with normal serum as a control for the antimeningococcic serum, and in duplicate in order to cover in a measure the variable response of the mice to the inoculation (Table

TABLE I.

Virulence Test of Regular Meningococcus 4.

Weight of mice.	Dose of suspension.	Result.
gm.	*cc.*	
17	0.5	D.* 13 hrs.
15.5	0.25	" 43 "
15	0.12	" 17 "
14	0.06	S. R.
14	0.03	D. 9 hrs.

* In the tables D. indicates died, S.R., sick and recovered, R., recovered.

II). The protocol brings out the facts (1) that the reaction of the mice to the inoculation is irregular, and (2) that normal horse serum exhibits no protective power against the meningococcus. Meningococci in large numbers were cultivated from the peritoneal cavity and heart's blood of the mice dying within 24 hours.

TABLE II.

Normal Horse Serum + Regular Meningococcus 4.

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot A.	Lot B.			Lot A.	Lot B.
gm.	*gm.*	*cc.*	*cc.*		
16	16	0.5	0.5	D. 14 hrs.	D. 9 hrs.
16	17	0.5	0.25	" 13 "	S. R.
15	15	0.5	0.12	" 9 "	D. 57 hrs.
14	15	0.5	0.06	" 37 "	R.
13	14	0.5	0.03	" 9 "	D. 85 hrs.

Two experiments were made with polyvalent antimeningococcic serum of high titer according to the standard based on agglutinin content (Tables III and IV). The serum also proved highly effective in the treatment of cases of epidemic meningitis. While Table III might be interpreted as giving evidence of protection, Table IV indicates an irregularity merely in the reaction of the mice, which cannot depend on protection. All the mice of Lot A which died yielded innumerable colonies of meningococcus from the peritoneal cavity and heart's blood.

TABLE III.

Antimeningococcic Serum + Regular Meningococcus 4.

Weight of mice.	Dose of serum.	Dose of suspension.	Result.
gm.	*cc.*	*cc.*	
18	0.5	0.5	D. 15 hrs.
17.5	0.5	0.25	" 15 "
16.5	0.5	0.12	S. R.
17	0.5	0.06	"
17	0.5	0.03	D. 9 hrs.

TABLE IV.

Antimeningococcic Serum + Regular Meningococcus 4.

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot A.	Lot B.			Lot A.	Lot B.
gm.	*gm.*	*cc.*	*cc.*		
15	17	0.5	0.5	D. 29½ hrs.	S. R.
16	18	0.5	0.25	" 21 "	"
16	15	0.5	0.12	" 24 "	D. 24 hrs.
14	15	0.5	0.06	" 29 "	S. R.
12	12	0.5	0.03	S. R.	"

The next tests were made with a parameningococcus and the same samples of normal and antimeningococcic horse serum.

Series II. Parameningococcus.

The chief point which the experiment brings out is the striking irregularity of the protection tests in this instance (Tables V to VII).

TABLE V.

Control, Parameningococcus G.

Weight of mice.	Dose of suspension.	Result.
gm.	*cc.*	
20	0.5	D. 27½ hrs.
16	0.25	" 25½ "
14	0.12	" 35 "
14	0.06	" 24 "
15	0.03	" 3½ "*

* Excluded; probable accident.

TABLE VI.

Normal Horse Serum + Parameningococcus G.

Weight of mice.	Dose of serum.	Dose of suspension.	Result.
gm.	*cc.*	*cc.*	
16	0.5	0.5	D. 16 hrs.
15	0.5	0.25	S. R.
15	0.5	0.12	D. 29½ hrs.
15	0.5	0.06	" 16 "

TABLE VII.

Antimeningococcic Serum + Parameningococcus G.

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot A.	Lot B.			Lot A.	Lot B.
gm.	*gm.*	*cc.*	*cc.*		
17	16	0.5	0.5	D. 23 hrs.	S. R.
16	15	0.5	0.25	S. R.	"
16	16	0.5	0.12	D. 6½ hrs.	D. 10½ hrs.
15	14	0.5	0.06	S. R.	S. R.
14	14	0.5	0.03	D. 17 hrs.	"

Series III. Intermediate Meningococcus.

The next test was carried out with an intermediate or irregular meningococcus tending slightly toward the regular (Tables VIII and IX). According to the agglutinin standard the polyvalent serum employed in this experiment was efficient; according to the protection test it is irregular in action and of practically no potency.

TABLE VIII.

Control, Intermediate Meningococcus 30.

Weight of mice.	Dose of suspension.	Result.
gm.	*cc.*	
15	0.5	D. 10½ hrs.
14	0.25	" 10½ "
14	0.12	S. R.
14	0.06	D. 34 hrs.
13	0.03	R.

TABLE IX.

Antimeningococcic Serum + Intermediate Meningococcus 30.

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot A.	Lot B.			Lot A.	Lot B.
gm.	*gm.*	*cc.*	*cc.*		
15	14	0.5	0.5	D. 8 hrs.	D. 10 hrs.
13	13	0.5	0.25	" 8 "	" 16 "
14	12	0.5	0.12	" 10 "	" 27½ "
13	13	0.5	0.06	" 8 "	" 16 "
12	12	0.5	0.03	" 29 "	S. R.

The final tests were carried out with two samples of monovalent antimeningococcic serum prepared in the horse, one for the normal or regular and the other for the parameningococcus. The horses yielding the two samples of serum had been under immunization for 12 and 9 weeks respectively. The agglutination titers of the sera at the time the protection tests were made was + to ++ at 1:500 dilution. According to the standard followed at The Rockefeller Institute the horses were not yet ready to yield therapeutic sera. The object was to ascertain whether they would show any evidence of protective power.

Series IV. Monovalent Sera.

In Tables X and XI Lots 1 and 4 refer to the number of the regular culture of meningococcus used for inoculation. According to Table XI the monovalent serum would have to be regarded as devoid of antibodies, which is obviously, in view of the agglutination titer, not the case.

Perhaps it would have been well to carry the suspensions to a greater dilution; namely, to doses of 0.06 and 0.03 cc. Since these tests were performed during the period last spring of acute mouse shortage, the smaller doses were omitted, especially as in the control tests the mice receiving 0.06 cc. both survived. The fact that of those given the monovalent serum one survived and the other died merely emphasizes the irregularity of this test.

TABLE X.

Control, Regular Meningococci 1 and 4.

Weight of mice.		Dose of suspension.	Result.	
Lot 1.	Lot 4.		Lot 1.	Lot 4.
gm.	*gm.*	*cc.*		
15	15	0.5	D. 30½ hrs.	D. 28 hrs.
15	15	0.25	" 28 "	" 36 "
15	15	0.12	S. R.	" 30 "
14	14	0.06	"	S. R.

TABLE XI.

Regular Monovalent Serum + Regular Meningococci 1 and 4.

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot 1.	Lot 4.			Lot 1.	Lot 4.
gm.	*gm.*	*cc.*	*cc.*		
15	17	0.5	0.5	D. 17½ hrs.	D. 11 hrs.
13	15	0.5	0.25	" 18 "	" 15½ "
13	14	0.5	0.12	S. R.	" 13 "

Tests with monovalent para serum and parameningococci were also carried out. Three parameningococcus cultures were employed (Tables XII and XIII). It is obvious from Tables XII and XIII that the tests give no indication of protective value in the serum in

TABLE XII.

Control, Parameningococci 60, 79, and 85.

Weight of mice.			Dose of suspension.	Result.		
Lot 60.	Lot 79.	Lot 85.		Lot 60.	Lot 79.	Lot 85.
gm.	*gm.*	*gm.*	*cc.*			
15	15	15	0.5	D. 9½ hrs.	D. 37 hrs.	D. 2½ hrs.*
15	15	15	0.25	" 31 "	" 37 "	" 33 "
14	14	14	0.12	" 31 "	" 31 "	" 3 " *
14	14	14	0.06	S. R.	" 33 "	" 31 "

* Excluded; accident.

TABLE XIII.

Monovalent Parameningococcic Serum + Parameningococci 60, 79, and 85.

Weight of mice.			Dose of serum.	Dose of suspension.	Result.		
Lot 60.	Lot 79.	Lot 85.			Lot 60.	Lot 79.	Lot 85.
gm.	*gm.*	*gm.*	*cc.*	*cc.*			
15	17	17	0.5	0.5	D. 11 hrs.	D. 11 hrs.	D. 19 hrs.
15	15	15	0.5	0.25	" 7 "	S. R.	" 11 "
15	14	16	0.5	0.12	S. R.	D. 13 hrs.	" 19 "
13	13	13	0.5	0.06	"	" 21 "	" 20 "

spite of a moderate content of agglutinin and doubtless of therapeutic principles.

The last two series of experiments were reversed so that the monovalent sera were made to react with their opposite cultures—the regular serum with para cultures and the para serum with regular cultures. Aside from what appeared to be an accidental survival, all the inoculated mice succumbed.

DISCUSSION.

In carrying out the experiments described in this paper no attempt was made to restudy, in an intensive manner, the general subject of the protective power of the antimeningococcic serum on animals. The purpose was merely to repeat the experiments of Hitchens and Robinson on which they base their argument for a protection standard for the antimeningococcic serum.

Our results failed wholly to confirm those of Hitchens and Robinson and conform to the earlier studies in indicating that infection tests carried out in laboratory animals with the meningococcus give extremely variable results, and that this factor of variability enters into and affects unfavorably the protection tests.

Investigators are generally agreed that the antimeningococcic serum possesses protective power against experimental meningococcic infection. This power is shown either by the survival of the inoculated and serum-treated animal or by reduction in the number of meningococci and increase in intensity of the phagocytosis within the peritoneal cavity. But not only are these reactions irregular

in their occurrence but they are confined within relatively narrow limits and may succeed only when the minimum lethal dose of the culture inoculated is not exceeded. Reactions of this order are not well suited for purposes of standardization.

CONCLUSIONS.

Experiments were made for the purpose of testing the reaction of protection against infection as a measure of potency of antimeningococcic serum.

The results of the experiments were extremely variable and bore no relation to the quality of the sera as determined by the period of immunization of the horses from which they were obtained, or the indications of efficiency based upon their employment in human cases of epidemic meningitis.

The results also failed entirely to conform to the agglutination titer of the sera tested and to be affected by the different type forms of the meningococci.

We regard the protective power for laboratory animals of the antimeningococcic serum as an unsuitable index of its value in human medicine and as inferior to the agglutination titer as a standard of potency.

[Reprinted from the Proceedings of the Society for Experimental Biology and Medicine, October 16, 1918, Vol. xvi, No. 1, pp. 3–5.]

A METHOD OF PRODUCING EXPERIMENTAL SHOCK.

By T. S. GITHENS, I. S. KLEINER, A. L. MEYER, and S. J. MELTZER.

(*From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.*)

In the numerous investigations on experimental shock there is no uniformity as to the method of inducing it. Some authors simply say "the animal was reduced to shock," without stating by what method it was induced or how long it took to induce it. Others claim that they have produced profound shock by continuous stimulation of sensory nerves, while other writers are quite positive that it is impossible to induce shock by this method. The method *par excellence* of producing shock seems to be the exposure of the abdominal viscera. Here again opinions differ. For instance, Erlanger and his coworkers say that in order to reduce blood pressure to 50 mm. mercury, an exposure and *manipulation* of the intestines lasting from two to five hours is necessary. In the studies of shock by Githens and Meltzer "the intestines and stomach were dislocated and *frequently handled.*" Wiggers on the other hand states that a more regular and certain circulatory failure is induced *when the intestinal loops are not manipulated.* Many investigators kept the animals under surgical anesthesia throughout the experiment or at least during the greatest part of it.

Generally the fall of blood pressure is the sole criterion of shock. In the studies of Githens and Meltzer the fall of blood pressure and also the disappearance of pain sense were taken as criteria. They studied these phenomena an hour or more after the discontinuation of ether. Of forty-two dogs, in fifteen the blood pressure did not reach a level below 95 mm. within five hours after opening the abdomen, and pain sense returned as soon as the animals came out of ether. In only thirteen dogs the blood pressure reached a level below 70 mm. within two and a half hours and there was no return of pain sense. In these thirteen dogs the original blood pressure was not high. In eight dogs the blood pressure sank to a lower level, but sensation of

pain returned when ether was discontinued. In six dogs sensation was lost while blood pressure was still above 95 mm. of mercury.

In experiments undertaken to throw light upon a certain problem in shock, we came across a method which seems to be effective in producing shock in every case and fairly early. The signs of shock were obtained with this method without exception in an unbroken series of experiments on seven dogs and nine cats. The method consists in repeated, strong compression with thumb and finger of the small gut, care being taken to avoid traction upon the mesentery. In all the animals the original blood pressure was fairly high. The strongest effect was a fall of blood pressure from 145 to 45 mm. within one hour and four minutes (cat). In all the animals a considerable fall of blood pressure was obtained within about an hour and fifteen minutes. It seemed that when the compression was produced during deep etherization the fall began early and was more profound and of longer duration. Whether or not etherization had been deep, loss of pain sense was noted early. In most instances the blood pressure rises moderately again when the animal is left for some time without ether and without squeezing of the gut. In one dog with original blood pressure of 160, the blood pressure which fell to 68 mm. rose after three hours to 120 mm. It is different, however, with the sensory shock. In not a single instance did the pain sense return at any time. This holds good even for cases in which the lid reflex was prompt.

The subject will require a great deal of detailed study. But we thought of putting our experiments on record on account of the value it may have for experimental shock and especially on account of its possible practical importance in human surgery. In abdominal surgery no care is taken to avoid compressing of the gut; on the contrary, it is often employed to achieve a definite end. On the other hand, traction on the mesentery is carefully avoided. In our experience traction on the mesentery rather favors some rise of blood pressure.

[Reprinted from the Proceedings of the Society for Experimental Biology and Medicine, October 16, 1918, Vol. xvi, No. 1, pp. 6–8.]

INFLUENCE OF MERE OPENING THE ABDOMEN, OF STATE OF SHOCK, AND OF SUBSEQUENT SECTION OF SCIATIC NERVE UPON THE BLOOD FLOW FROM THE FEMORAL VEIN IN CATS.

By T. S. GITHENS, I. S. KLEINER, A. L. MEYER, and S. J. MELTZER.

(*From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.*)

From a series of experiments performed for the purpose of solving a certain problem in shock, a few definite facts were selected to be put on record. The following results were obtained from experiments on fourteen cats. The rate of the blood flow from the femoral vein shortly before opening the abdomen was taken as the unit; *i.e.*, 100 per cent. The figures indicate the time required for the flow of the same amount of blood, and hence express an inverse ratio to the rate; *e.g.*, 200 per cent. indicates that the rate was half as fast.

Influence of Opening the Abdomen.—The average time of flow from the femoral vein shortly after opening the abdomen in the fourteen cats is 136 per cent., that is, an average decrease of 36 per cent. in the rate. A decrease in rate of flow occurred in every experiment but one. The maximum decrease (in one cat) was 84 per cent. This result means that after the mere exposure of the abdominal viscera (before any change in volume and composition of the blood could have taken place) a slowing of the flow from the extremities takes place. In interpreting this fact the statement of H. Fischer, the first writer on shock from a physiological point of view, is worth considering; namely, that a certain degree of engorgement of the veins of the abdominal viscera can be observed immediately after opening the abdomen. This occurrence may be the cause of the slowing of the flow from the veins of the extremities. As far as we know these facts have not been observed or discussed by any experimental or surgical writers. It may be mentioned that there was a variable but generally a moderate fall of blood pressure immediately after opening the

abdomen; but there was no definite proportion between the fall of blood pressure and the decrease in the rate of flow.

The Rate of Blood Flow in Shock.[1]—When the blood pressure had fallen to about 70 mm. of mercury, the animals were considered in shock; in the majority of cases it fell to 60 mm. or less; the lowest pressure reached was 30 mm. In every cat the blood flow in shock was slower than just after opening the abdomen. On the average it was 301 per cent. of the original. The decrease in the rate of flow seemed in a general way to follow the degree of fall of blood pressure, but was not absolutely proportional.

Influence of Cutting the Sciatic Nerve upon Blood Flow in Shock.—After section of the sciatic nerve the rate of blood flow increased markedly in eleven cats, slightly in two and in one there was a slight decrease. The average increase over the flow in shock was about one third, that is, the time of flow decreased from 301 to 196 per cent., but the average rate was still only about half as fast as the original flow (100 per cent.). In five cats the flow became as fast (or even faster) as immediately after opening the abdomen; and in one of these five the flow was as fast as the original. The blood pressure rose slightly on section of the nerve in eleven cats. The remaining three were those in which there was little or no increase in flow.

The fact that in these experiments the blood flow after section of the sciatic nerve in shock is usually far below normal, and never above normal, certainly does not support the assumption, now current, that vaso-constriction is greater in shock than in normal conditions of the animal.

[1] In the present series of experiments the blood obtained from the femoral vein was mixed with a small amount of sodium citrate and reinjected through the jugular vein. Hemorrhage therefore was not a factor in the production of shock in these experiments.

[Reprinted from The Journal of Pharmacology and Experimental Therapeutics, November, 1918, Vol. xii, No. 4, pp. 211–214.]

THE APPLICATION OF A CONCENTRATED SOLUTION OF MAGNESIUM SULPHATE TO SCALDS AND BURNS.

By S. J. MELTZER.

(From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.)

(Received for publication, August 20, 1918.)

I wish to place on record results of experiments with external application of a concentrated solution of magnesium sulphate carried out several years ago; the experiments were repeated since and similar results were obtained. My original experiments were briefly mentioned by Dr. Reid Hunt in a discussion of a paper of S. Solis-Cohen (1). At the period when the discussion took place we published numerous experiments on the action of magnesium sulphate on the nervous system; I abstained therefore from publishing my experiments on the local application of $MgSO_4$ on burns, etc., to avoid giving the impression that I consider magnesium sulphate as a panacea for all ills. We are engaged at present in experimental studies on the value of local application of a concentrated solution of magnesium sulphate to injuries caused by other procedures than scalding. I therefore decided to publish now my original experiments with hot water, presenting at the same time characteristic photographs illustrating the obtained results.

The experiments were made on non-injured and non-shaved rabbits' ears. The procedure was as follows. The animal was stretched out on a Cannon board with the head appropriately elevated so that glasses containing solutions could be put under the ears. The animal was deeply anesthetized, both ears were pulled down through the anterior opening of the board and about one-third of them was submerged for a short period in hot water. Then the water was removed and one ear was submerged in a 25 per cent solution of epsom salt (1 mol. $MgSO_4$ + 7 mol. of water) and the other ear was submerged in a solution of NaCl (either 0.9 or 6 per cent). The ears

were kept submerged in these solutions for about two or three hours, the solutions being changed two or three times during this period.

A number of experiments had to be made to previously establish the degree of temperature of the water and the length of time for the submersion of the ears in it which were necessary to bring out that degree of inflammation which could permit a doubtless conclusion as to the favorable action of the solution of magnesium salts. It was found that the inflammations brought about by water of a tem-

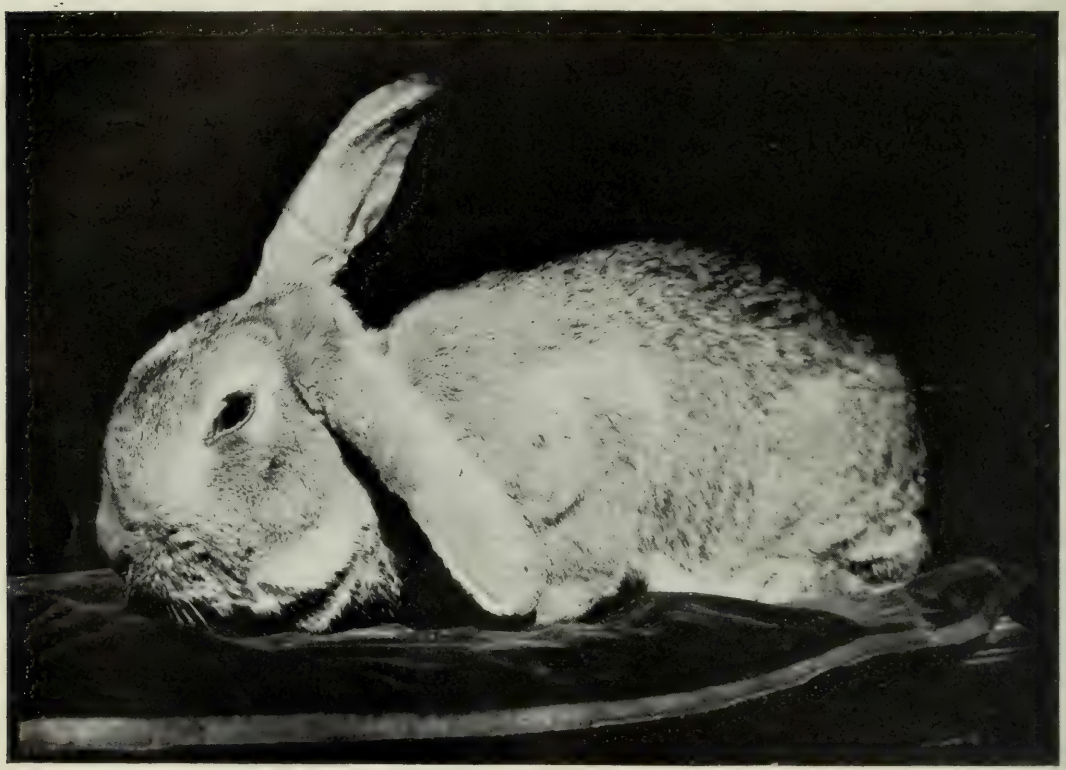


FIG. 1.

perature less than 56°C. were too insignificant to permit a statement as to the effect of $MgSO_4$; the difference between the two ears was too small and the recovery was fairly rapid and complete in both ears. A submersion in water of a temperature above 63°C., for a three minute duration or over, brought out such a degree of burn, with a subsequent destruction, of the submerged part of the ear, which did not permit the drawing of a definite conclusion as to the retarding effect of the submersion in magnesium sulphate solution. (It should

be noted here that during the submersion of the ears the temperature of the water becomes fairly rapidly reduced.)

The experiments with submersion in water of temperatures lying between the two above mentioned degrees have always shown that the inflammation of the ear which was submerged in $MgSO_4$ was undoubtedly retarded or nearly completely prevented. However, for

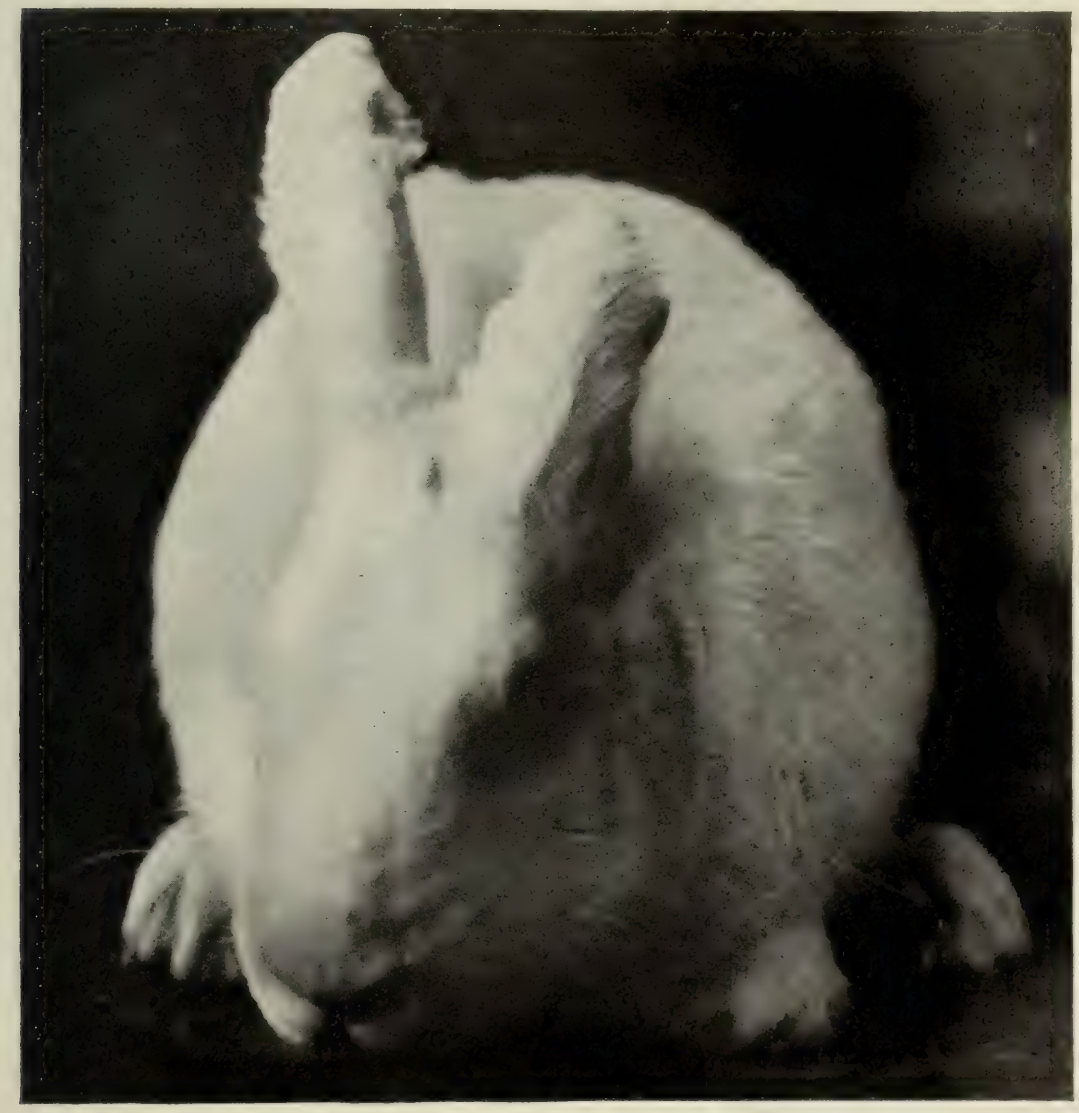


Fig. 2.

the purpose of obtaining photographic evidence of the usefulness of $MgSO_4$ the temperature of the water had to be above 60°C. and the duration of the submersion in it could not exceed two minutes. The obtained results are well expressed by the reproduction of the two figures.

Figure 1 is from a photograph taken about three hours after the ears were removed from both solutions in which they were submerged for two hours. The right ear was kept in a 25 per cent solution of $MgSO_4$ and the left ear in a solution of NaCl 0.9 per cent. They were submerged in these solutions immediately after the removal from the hot water. The latter had an original temperature of 62°C. and the ears were kept in the water for *two minutes*. The right ear ($MgSO_4$) was erect and appeared to be normal; the left ear (normal saline) was swollen, heavy, hot and red.

The ears of the rabbit in figure 2 were kept for *three minutes* in water of an original temperature of 62.5°C. The right ear was submerged in a 25 per cent solution of $MgSO_4$ and the left ear in a 6 per cent solution of NaCl, for three hours. The photograph was taken sixteen days later. Of the left (NaCl) ear about one-third sloughed away and the stump was healing. On the right ear there was some sloughing at the tip which was in process of healing.

I have had occasions to see cases of burns in human beings. First and second degrees of burns are invariably arrested in their development when molecular solutions of $MgSO_4$ have been applied early. Third degrees of burns run as a rule a more favorable course under application of $MgSO_4$ than under any other treatment. Higher concentrations than 25 per cent seem to exert a still better influence. The favorable action of the application of $MgSO_4$ in advanced stages of burns of second and third degree is less striking, especially on account of the infection present; but even in this stage it seems to exert a favorable influence and ought to be used either in combination, or alternatingly, with antiseptics.

REFERENCE.

(1) Solis-Cohen, S.: Jour. Amer. Med. Assoc., liii, p. 1892.

[Reprinted from The Journal of Biological Chemistry, August, 1918, Vol. xxxv, No. 2, pp. 281–283.]

REDUCTION OF ALDEHYDES TO CORRESPONDING ALCOHOLS.

I. Reduction of Heptylic Aldehyde (Oenanthol).

By P. A. LEVENE and F. A. TAYLOR.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 26, 1918.)

Two methods are in vogue for the reduction of aldehydes into alcohols. In one the reduction is accomplished by means of zinc dust and is carried out in a solution of glacial acetic acid. The second consists in the use of sodium amalgam. The disadvantage of the first method lies in the time required for the completion of the reaction, and in the fact that the alcohol formed by this process combines with the acetic acid and is isolated in form of the corresponding acetate.

The reduction with sodium amalgam was first carried out in an aqueous solution. This condition was found unsatisfactory because of the low degree of solubility of the higher aldehydes in it. The process was subsequently modified and glacial acetic acid was employed as solvent. Through this modification the yield of the alcohol was somewhat improved. However, part of the alcohol formed an ester with the acetic acid. The yield was approximately 40 per cent of the theory.

Very recently the reduction of aldehyde to alcohol was accomplished through the process of hydrogenation, by means of hydrogen in the presence of colloidal platinum. This method from the standpoint of yield was very satisfactory. It is, however, troublesome when the reduction of large quantities of aldehyde is desired.

Because some imperfection is attached to every one of the existing methods an attempt was made to reduce aldehydes directly by means of metallic sodium. The process was essentially the same as employed in this laboratory for the reduction of esters to the corre-

sponding alcohols. A fine emulsion of the metal in toluene was made and glacial acetic acid was used as a solvent for the aldehyde.

The apparatus for the reduction was the same as described by Levene and Allen for the reduction of esters. The details of the operation are described below. Here it suffices to mention that the reaction is completed within 1 hour and that the alcohol remains uncombined with the acetic acid. The yield is about 60 per cent, which is higher than the one obtained either by means of zinc dust or by sodium amalgam.

The method will be extended to the reduction of other aldehydes both of the aliphatic and of the aromatic series.

EXPERIMENTAL.

N-Heptylic Alcohol.—Oenanthol, which boiled between 152 and 156°, was reduced in 30 gm. lots in the apparatus described by Levene and Allen for the reduction of esters with sodium and alcohol. Four equivalents of sodium were suspended in 100 cc. of dry toluene, and the mixture was heated. When the sodium was melted, the stirrer was rotated vigorously in order to form a fine emulsion. The bath was then removed and the flask quickly cooled in ice, the stirring being continued.

The oenanthol, mixed with 50 cc. of glacial acetic acid and 50 cc. of toluene, was then introduced through a dropping funnel at such a rate that there was no refluxing. The mixture was continuously cooled and stirred. In order to complete the reduction and dissolve all the sodium, 50 to 75 cc. of glacial acetic acid were next introduced in the same manner. The whole operation required about an hour.

When the reduction was finished, the flask was removed and treated with water to dissolve the sodium acetate, and the layers were separated. The aqueous solution was saved for treating the next reduction product as, at times, the reaction product contained some undissolved sodium. This solution could be used safely instead of alcohol for completing the solution of the excess sodium.

The toluene layers were united and distilled with steam. The first distillate, mainly toluene and acetic acid, was collected separately. The distillation was continued as long as the odor of heptylic alcohol

persisted. The toluene distillate was fractionated and gave 15 per cent of the total yield of heptylic alcohol.

The oily residue at the end of the distillation gave no further alcohol or acid after saponification with potassium hydroxide.

The combined aqueous solutions obtained from the reductions contained no heptylic alcohol; therefore they were discarded.

The yield of heptylic alcohol from 360 gm. of oenanthol was 208 gm., or 58 per cent of the theoretical. The substance boiled at 173–177°C. On redistillation, the boiling point was 175.5–176.5°C. (corrected).

$$d_4^{20} = 0.8254, \quad n_D^{20} = 1.42463$$

0.1010 gm. substance gave 0.2686 gm. CO_2 and 0.1240 gm. H_2O.

	Calculated for $C_7H_{16}O$: *per cent*	Found: *per cent*
C	72.41	72.52
H	13.77	13.74

N-Heptyl Phenylurethane.—3 gm. of heptylic alcohol and 3 gm. of phenylisocyanate were heated at 175°C. for a few minutes, cooled, and the solid product was recrystallized from dilute alcohol. After four recrystallizations the melting point was constant at 60°C. (corrected). It crystallizes in needles.

0.2000 gm. substance neutralized 8.49 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{14}H_{21}O_2N$: *per cent*	Found: *per cent*
N	5.95	5.94

[Reprinted from The Journal of Biological Chemistry, August, 1918, Vol. xxxv, No. 2, pp. 285–290.]

CEPHALIN. V.

Hydrocephalin of the Egg Yolk.

By P. A. LEVENE and C. J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 28, 1918.)

Thudichum[1] who discovered cephalin represented its composition in the following manner:

$$O = P \begin{cases} C_{17}H_{28}O_3 \text{ (cephalyl)} \\ C_{18}H_{35}O_2 \text{ (stearyl)} \\ C_3H_7O_3 \text{ (glyceryl)} \\ C_5H_{11}ON \text{ (neuryl)} \end{cases} \quad C_{43}H_{81}NPO_9$$

The various samples of cephalin analyzed by him had an elementary composition which differed from that required by theory.

	Calculated:	Found:
C	65.59	60.00
H	10.38	9.39
N	1.78	1.68
P	3.94	4.27

The composition of the substance led to an empirical formula of $C_{42}H_{69}NPO_{13} + 5H_2O$. Analyzing the discrepancy of the theoretical and empirical composition of the substance Thudichum writes:

"But the assumption of the presence of five molecules of water of hydration brings the constituent hydrogen to so low a figure that it cannot be explained out of the sum of the chemolytic products. On the other hand the assumption of the presence of five atoms of loosely attached oxygen meets also with theoretical objections. The difficulty here touched requires evidently experimental elucidation."

---

[1] Thudichum, J. L. W., A treatise on the chemical constitution of the brain, based throughout on original researches, London, 1884, 57, 58.

Subsequent workers have modified the view of Thudichum on the nature of two of the components of cephalin. Parnas[2] has demonstrated that cephalinic acid is an unsaturated normal octadecylic acid, and assumed it to belong to the diolefin series. Baumann[3] and Renall[4] have shown that the basic substance of cephalin has the structure of amino ethyl alcohol. With this modification the theory requires the following composition of cephalin.

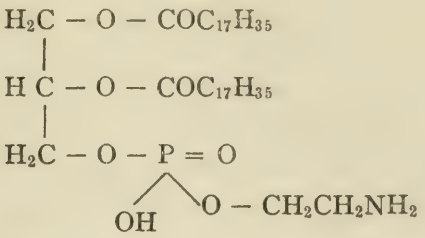
C	H	N	P
per cent	*per cent*	*per cent*	*per cent*
66.16	10.57	1.88	4.18

This composition differs from the empirical even more than the one required by Thudichum's theory. None of the subsequent workers on cephalin have made any serious effort to explain the discrepancy. Our own[5] efforts to prepare a sample of cephalin or a derivative of it, having the composition required by theory, were not very successful.

Yet it is important to bear in mind that nearly all samples of cephalin described by various workers possessed practically identical compositions. Hence, one faces the following dilemma: Either cephalin as generally prepared contains a component which up till the present has escaped identification, or it contains a constant impurity. Recently Levene and West[6] have shown that dehydrolecithin, having a composition required by theory, still contained about 20 per cent of its nitrogen in the form of amino nitrogen. In this fact they saw an indication of the possibility of isolating cephalin having the theoretical composition. This expectation has now been realized. From material prepared in the manner described in the previous article, which was a mixture of reduced lecithin and cephalin, hydrocephalin has been obtained which possessed the composition required by theory, and which had in one sample 93 per cent of its total nitrogen in form of amino nitrogen and in another 97 per cent.

[2] Parnas, J., *Biochem. Z.*, 1909, xxii, 411.
[3] Baumann, A., *Biochem. Z.*, 1913, liv, 30.
[4] Renall, M. H., *Biochem. Z.*, 1913, lv, 296.
[5] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 41; xxv, 517.
[6] Levene and West, *J. Biol. Chem.*, 1918, xxxiv, 175.

Thus the structure of reduced cephalin may be regarded as established and expressed as follows.



The reason for the difficulty in obtaining the non-reduced substance having a theoretical composition still remains obscure.

Assuming cephalinic acid to contain three double bonds and further assuming that each molecule of the substance precipitated in the usual way contains four and a half molecules of water adhering to it, one obtains a calculated composition which does not differ essentially from the empirical.

	C *per cent*	H *per cent*	N *per cent*	P *per cent*
Calculated	60.54	10.54	1.72	3.82
Found (averages)	60.31	9.50	1.80	3.70

Thus far there exists no other experimental evidence for or against this supposition. However, the fact that reduced cephalin of a theoretical composition is obtainable proves that cephalin itself possesses essentially the structure generally assumed for it.

A point in the structure of cephalin which is still under discussion is that of the structure of the glycerophosphoric acid contained in it. Frankel and Dimitz have claimed that the glycerophosphoric acid in cephalin differs from that of lecithin, the latter rotating to the left, the former to the right. It was found that hydrocephalin had an optical rotation in the same direction and practically of the same magnitude as hydrolecithin. In the reduced cephalin as in the reduced lecithin an unsymmetric carbon atom may arise only in the glycerophosphoric acid when the phosphoric acid is attached to one of the terminal carbon atoms. Reduced cephalins and lecithin differ only in that the base in one is amino ethyl alcohol and in the other choline. It is hard to believe that these two substances so closely related structurally and possessing practically the same optical rotation should be derived from two enantiomorphous glycerophosphoric acids.

Attempts to isolate the glycerophosphoric acid from cephalin are now in progress in this laboratory.

EXPERIMENTAL.

The starting material was in part the same which was described by Levene and West in a previous article and in part another lot prepared in the identical manner. The previously described sample contained 20 per cent of its total nitrogen in the form of amino groups. The new material (No. 402) contained 13.09 per cent.

The separation was completed in three phases. The first phase aimed to remove the traces of unsaturated material that might adhere to the substance. This step is accomplished by dissolving the crude material in chloroform and by pouring the solution into a large excess of dry ether. The analysis of the product obtained by this purification (No. 439) is reported in the accompanying table.

The second step accomplished a concentration of the cephalin part so that the mixture consisted approximately of equal parts of the two components. This phase was arrived at through repeated fractional crystallizations out of a solution consisting of equal parts by volume of chloroform and ethylmethyl ketone; 1 part of air-dry material is dissolved in 15 parts of the solvent. As a rule four crystallizations suffice to achieve this phase.

The final purification is accomplished by the recrystallization of the partially purified material out of a solution consisting of 1 part of chloroform and of 2 parts of ethyl alcohol (99.5 per cent); 1 part of the air-dry material is dissolved in 16 parts of the solvent. The crystallization is allowed to proceed at room temperature, and care is taken to prevent evaporation of the chloroform. Out of the mother liquor on standing at about 10°C. a second precipitate is formed which contains about 50 per cent of cephalin. The mother liquor of this material on standing at room temperature in an open flask loses part of its chloroform, and then gives rise to a precipitate which contains only a low proportion (14.86 per cent) of cephalin.

The newly prepared sample will be designated Sample A, and the older one Sample B.

Sample A.

Phase I, No. 402.

0.1034 gm. substance gave 0.2474 gm. CO_2, 0.1034 gm. H_2O, and 0.0108 gm. ash.

0.1938 " " required for neutralization 3.43 cc. 0.1 N acid.

0.2903 " " gave 0.0424 gm. $Mg_2P_2O_7$.

A sample of the material contained 0.03791 gm. total N and 0.00497 gm. NH_2-N.

Phase II, No. 442.

0.1004 gm. substance gave 0.2422 gm. CO_2 and 0.098 gm. H_2O.

0.1912 " " required for neutralization 2.83 cc. 0.1 N acid.

0.2868 " " gave 0.0410 gm. of $Mg_2P_2O_7$.

A sample of the material contained 0.03842 gm. total N and 0.01133 gm. NH_2-N.

No. 459.

0.0998 gm. substance gave 0.2410 gm. CO_2 and 0.0962 gm. H_2O.

0.1912 " " required for neutralization 2.73 cc. 0.1 N acid.

0.2868 " " gave 0.0414 gm. $Mg_2P_2O_7$.

A sample of the material contained 0.02078 gm. total N and 0.007623 gm. NH_2-N.

No. 463.

0.0956 gm. substance gave 0.2310 gm. CO_2, 0.0938 gm. H_2O, and 0.0094 gm. ash.

0.2000 " " required for neutralization 2.73 cc. 0.1 N acid.

0.3000 " " gave 0.0436 $Mg_2P_2O_7$.

A sample of the material contained 0.0354 gm. total N and 0.01771 gm. NH_2-N.

Phase III, No. 465.

0.1058 gm. substance gave 0.2520 gm. CO_2, 0.0992 gm. H_2O, and 0.0102 gm. ash.

0.1940 " " required for neutralization 2.73 cc. 0.1 N acid.

0.2910 " " gave 0.0448 gm. $Mg_2P_2O_7$.

A sample of the material contained 0.01204 gm. total N and 0.01124 gm. NH_2-N.

Filtrates from No. 465.

No. 547. A sample of the material contained 0.01344 gm. total N and 0.001998 gm. NH_2-N.

$$[\alpha]_D^{20} = \frac{+ 0.30 \times 100}{0.5 \times 10} = + 6.0$$

Sample B.

Phase I was reported in a previous paper.

Phase II, No. 557.

0.1054 gm. substance gave 0.2612 gm. CO_2, 0.0954 gm. H_2O, and 0.1059 gm. ash.

0.1932 " " required for neutralization 2.53 cc. 0.1 N acid.

0.2898 " " gave 0.0393 gm. $Mg_2P_2O_7$.

A sample of the substance gave 0.008064 gm. total N and 0.005255 gm. NH_2-N.

$$[\alpha]_D^{20} = \frac{+ 0.30 \times 100}{0.5 \times 10} = + 6.0$$

Phase III, No. 559.

0.0996 gm. substance gave 0.2386 gm. CO_2, 0.0934 gm. H_2O, and 0.0100 gm. ash.

0.1934 " " required for neutralization 2.67 cc. 0.1 N acid.

0.2901 " " gave 0.0403 gm. $Mg_2P_2O_7$.

A sample of the material gave 0.01383 gm. total N and 0.1361 gm. NH_2-N.

$$[\alpha]_D^{20} \text{ in chloroform} = \frac{+ 0.30 \times 100}{1 \times 5} = + 6.0$$

	Phase of purification.	C	H	N	P	$\frac{NH_2\text{-N}}{\text{Total N}}$	Rotation $[\alpha]_D^{20}$
		per cent	*per cent*	*per cent*	*per cent*		
Sample A.	I No. 402	65.25	10.70	2.13	4.07	13.09	
	II_1 " 442	65.79	10.91	2.08	3.98	31.60	
	II_2 " 459	65.86	10.81	2.00	4.02	36.59	
	II_3 " 463	65.89	10.98	1.91	4.05	50.00	
	III " 465	65.0	10.50	1.97	4.29	93.02	
	" 547					14.86	+6.0
Sample B.	I	65.50	11.30	1.80	3.90	20.00	
	II " 556	65.26	10.32	2.01	3.97	65.19	+6.0
	III " 559	65.33	10.50	1.94	3.87	98.33	+6.0
Theory for reduced cephalin, $C_{41}H_{82}O_8NP$.....		65.80	11.05	1.87	4.15	100.00	

[Reprinted from The Journal of Biological Chemistry, October, 1918, Vol. xxxvi, No. 1, pp. 73–87.]

EPIMERIC HEXOSAMINIC ACIDS.

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 29, 1918.)

The synthesis of α-hexosaminic acids had for its principal aim the allocation of the NH_2 group in the natural and synthetic aminohexoses.

Irvine and Hynd[1] have shown that depending on conditions glucosamine may be converted either into glucose or into mannose. Levene and La Forge have shown that choosing the proper conditions one may obtain from glucosamine either anhydrogluconic or anhydromannonic, either anhydrosaccharic or anhydromannosaccharic acids. Hence there was no foundation for the preference for either one of the two alternative configurations for glucosamine.

Since at present for the purpose of proving the configuration of the amino sugars there exists no direct chemical means such as Fischer and his coworkers employed for developing the structure of nitrogen-free sugars, one has to resort to methods of analogy.

It was hoped that a comparison of the properties of pairs of epimeric hexonic acids with corresponding pairs of hexosaminic acids might offer some suggestions for the allocation of the amino group. The properties which are compared in the present discussion are: first, the equilibrium of the two epimers formed on the synthesis of the hexonic acids from the corresponding pentoses; second, the direction of the rotation of the α-carbon atom in corresponding pairs of epimers. An attempt to compare these properties had already been made by the present writer at a time when pairs of epimeric hexosaminic acids had not yet been known.

In the present communication the properties of three pairs of epimeric α-hexosaminic acid are compared. Designated by the parent pentose the substances are: two arabinohexosaminic acids,

[1] Irvine, J. C., and Hynd, A., *J. Chem. Soc.*, 1912, ci, 1128; 1914, cv, 698. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 351.

two lyxohexosaminic acids, and two xylohexosaminic acids. Of the first pair one can be prepared from glucosamine, occurring in nature, or synthetically from arabinose. The substance employed in the present investigation was prepared by oxidation of glucosamine. The epimeric acid was prepared by the action of pyridine on glucosaminic acid.

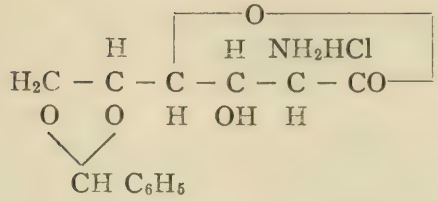
Of the second pair also, one may be obtained from the naturally occurring sugar chondrosamine as well as by synthesis from lyxose. In the present work the substance was obtained by both methods. The acid epimeric to chondrosaminic was prepared synthetically. The third pair was prepared synthetically from xylose.

Both the pyridine and the synthetic method always furnished a mixture of the two epimeric substances and in every substance the separation was accomplished by means of fractional crystallization either out of water or out of dilute ethyl or methyl alcohol.

It was fortunate that one member was obtainable in a condition of undisputed purity in every one of the three pairs. The purity of glucosaminic and chitosaminic acids is to be accepted by reason of their preparation from naturally occurring sugars. One, the dextroxylohexosaminic acid, forms a crystalline lactone and this was converted into a pure acid. Also, fortunately it was possible to obtain epiglucosaminic acid in a state of unquestionable purity. Under certain conditions out of a mixture of the two only one, namely the epiglucosaminic acid, formed a crystalline lactone.

Data obtained on this pair of epimers was of great assistance in the separation of the individual members of the other two pairs; namely, on this pair the numerical value of the optical rotation of the α-carbon atoms was established. This value was then employed as a standard of purity in the separation of the individual members of the other two pairs. Indeed, when this value was obtained further recrystallization remained without influence on the rotation of the amino-acid.

Later in the work the levoxylohexosaminic acid was obtained from a crystalline derivative of the acid; namely, from the benzal xylohexosaminic lactone hydrochloride.



This substance yielded an acid possessing the same optical rotation as the parent substance. Thus by fractional crystallization alone the complete separation of the epimers was accomplished.

The identity of the individual amino-acids was further demonstrated by results by deaminization experiments. These results will be communicated in a separate publication.

Turning them to the principal point of the investigation, namely to the comparison of the properties of epimeric amino-acids amongst themselves and with the corresponding members of non-nitrogenous sugar acids, we find that in each pair of hexosaminic as in each pair of hexonic acid the rotation of the α-carbon atom of one member is to the right and the other to the left.

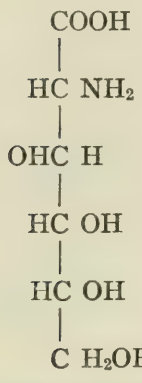
Incidentally it is to be noted that the numerical value of the rotation of the α-carbon in all six hexosaminic acids is practically constant. In the series of hexonic acids those rotating to the right are gluconic, galactonic, gulonic.

Turning their attention towards the equilibrium of the products of condensation of pentoses with prussic acid,[2] Fischer and his coworkers found that arabinose yields in preponderance mannonic acid, which is levorotary; lyxose and xylose yield galactonic and gulonic acid, respectively, which are dextrorotary.

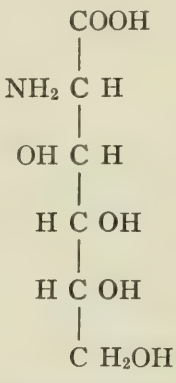
In the action of prussic acid on amino-pentosides, arabinose yields principally the levorotary acid, lyxosamine and xylosamine both yield in preponderance a dextrorotary acid. Thus there seems to exist a complete analogy in the predominating products formed from the pentoses with those from the amino-pentosides, from the view-point of the properties of their α-carbon atoms. May not, therefore, there exist also a similarity in their configuration? For the present this is only a suggestion. If this view should find further

[2] Fischer, E., *Ber. chem. Ges.*, 1890, xxiii, 2611, 2625. Fischer, E., and Bromberg, O., *ibid.*, 1896, xxix, 581.

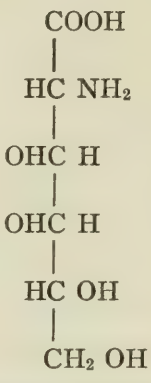
corroboration then the configuration of the six known hexosaminic acids will be expressed as follows:



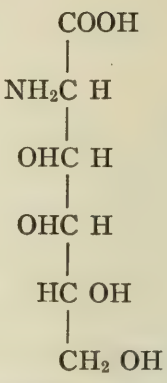
d-epichitosaminic (α-amino-gluconic)



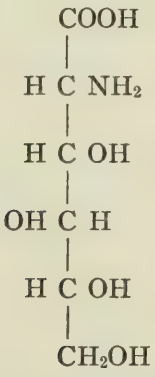
d-chitosaminic (α-amino-mannonic)



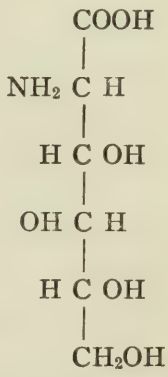
d-epichondrosaminic (α-amino-galactonic)



d-chondrosaminic (α-amino-talonic)



d-dextroxylohexosaminic (α-amino-gulonic)



d-levoxylohexosaminic (α-amino-idonic acid)

In a previous publication[3] identical conclusions were reached in regard to chitosaminic and chondrosaminic acids, whereas in regard to the derivatives of xylose the conclusion was contradictory and was evidently erroneous. The fact that in the earlier experiment after the action of pyridine an acid was obtained with a higher dextrorotation than the original is explained simply by the fact that in the presence of impurities only the more insoluble substances crystallized, while the more soluble remained in the mother liquor.

[3] Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 145. Levene, P. A., and Meyer, G. M., *ibid.*, 1916, xxvi, 355; 1917, xxxi, 627.

It would seem more rational to abandon the term glucosamine and glucosaminic acid and to return to the older terms chitosamine and chitosaminic acid.

The respective optical rotations and the numerical values of the rotations of the α-carbon atoms are as follows:

Acid.	$[\alpha]_D^{20}$	B–A	$\frac{B-A}{2}$
A *d*-Chitosaminic	−15	25	12.5
B *d*-Epichitosaminic	+10		
A *d*-Chondrosaminic	−17	25	12.5
B *d*-Epichondrosaminic	+8		
A *d*-Levoxylohexosaminic	−11	25	12.5
B *d*-Dextroxylohexosaminic	+14		

This table contains the best evidence as yet advanced in support of van't Hoff's optical superposition theory. In the series of α-hexosaminic acids the law holds true not only in regard to the direction of the rotation but also in regard to its numerical value. The reason for the more perfect agreement between the numerical values in this series of substances may be attributed to the fact that the phenomena of electrolytic dissociation here play a comparatively subordinate part, and that individual amino sugar acids are accessible in purer state, since the separation of their epimers is more perfect than those of the ordinary sugar acids.

EXPERIMENTAL.

Epichitosaminic Lactone Hydrochloride.

100 gm. of chitosaminic acid are taken up in 1,000 cc. of water to which 100 gm. of pyridine are added and heated in an autoclave at 105°C. for 4 hours. The solution is then evaporated under diminished pressure to a small volume, so that the greater part of chitosaminic acid crystallizes in the distillation flask. The residue is again

brought into solution with a minimum amount of boiling water. To the solution while still hot an equal volume of 95 per cent alcohol is added. The solution then becomes filled with crystals of chitosaminic acid. To complete the crystallization the material is allowed to stand from 8 to 12 hours.

The filtrate is then concentrated under diminished pressure (the temperature of the water bath should not exceed 50°C.) to a small volume. To this, hot 95 per cent alcohol is added until the solution begins to show opalescence, and enough hot water is then added to clarify the solution. On standing, more readily after some scratching, a crystalline substance begins to deposit. The deposit reaches its maximum in about 24 hours. This substance is dissolved in a minimum amount of hot water, boiled with charcoal, and the filtrate treated with 95 per cent alcohol in the manner just described. The crystalline substance obtained is at times nearly colorless, and at times of very light tan. It consists of a mixture of varying proportions of chitosaminic and epichitosaminic acids. On one occasion it consisted of pure chitosaminic acid. The optical rotation of the substance varied from $[\alpha]_D^{20} = +3.0°$ to $+8.0°$, and on one occasion it had $[\alpha]_D^{20} = +10.0°$.

For further purification the epichitosaminic acid was converted into its lactone. This was accomplished in the following way: to 3 gm. of the acid 2 cc. of 99.5 per cent alcohol and 1 cc. of benzaldehyde were added, and dry hydrochloric acid gas passed. The acid at first goes into solution and subsequently a crystalline deposit begins to reappear. The treatment with the gas is continued approximately 7 minutes. After standing 8 to 12 hours, 3 cc. of dry ether (dried over sodium) were added, and the material was allowed to stand an additional 12 or 24 hours in order to obtain the maximum yield of the lactone hydrochloride. The addition of benzaldehyde has for its purpose the conversion of the chitosaminic acid into the benzal of its ethyl ester. Under these conditions it remains in solution and permits the crystallization of the epichitosaminic acid lactone. In the absence of benzaldehyde an amorphous precipitate is formed which probably consists of a mixture of the two lactones.

The lactone is crystallized out of methyl alcohol. For this purpose it is advisable to dissolve the crude substance in a large excess

of hot methyl alcohol, decolorize the solution with charcoal, and then concentrate it to a small volume. The lactone crystallizes in form of colorless prismatic needles. The substance had a melting point = 203°C. (with gas evolution). Its composition was as follows:

0.1040 gm. substance gave 0.1286 gm. CO_2 and 0.1040 gm. H_2O.
0.1000 " " required for neutralization 4.65 cc. 0.1 N acid.
0.1000 " " " 4.50 cc. 0.1 N $AgNO_3$.

	Calculated for $C_6H_{12}NO_5Cl$: *per cent*	Found: *per cent*
C	33.71	33.72
H	5.67	5.47
N	6.55	6.51
Cl	16.25	15.97

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{+ 0.90 \times 100}{1 \times 2} = + 45.0°$$

Epichitosaminic Acid.

2 gm. of the lactone were dissolved in 20 cc. of distilled water and the solution was rendered alkaline by means of a solution of barium hydroxide. The solution was allowed to stand over night, then the barium was removed quantitatively by means of sulfuric acid, the hydrochloric acid by silver carbonate, and the excess of the latter reagent by hydrogen sulfide. The mother liquor of the silver sulfide was concentrated to a small volume; on addition of little alcohol the epichondrosaminic acid crystallizes immediately in prismatic needles. The substance had a melting point of 198°C. (uncorrected) with gas evolution.

The composition of the substance was the following.

0.0992 gm. substance gave 0.1336 gm. CO_2 and 0.0606 gm. H_2O.

	Calculated for $C_6H_{13}NO_6$: *per cent*	Found: *per cent*
C	36.92	36.80
H	6.66	6.72

The optical rotation of the substance was

Initial.
$$[\alpha]_D^{20} = \frac{+ 0.20 \times 100}{1 \times 2} = + 10°$$

Equilibrium.
$$[\alpha]_D^{20} = \frac{+ 0.78 \times 100}{1 \times 2} = + 39°$$

The rotation of chitosaminic acid being $-15.02°$C., that of the α-carbon atom is $\frac{10.0 - (-15.02)}{2} = 12.51°$C.

Synthesis of Amino Sugar Acids.[4]

In the earlier work of Levene and La Forge the conditions given by Fischer and Leuchs for the synthesis of chitosamine were followed. In the subsequent work, however, it was found that the reaction was very capricious and the results fluctuating. Thus for a long time the maximum yield of xylohexosaminic acid was 10 per cent, and a certain number of experiments ended in complete failure. However, after some experimentation it was discovered that the result of the reaction of prussic acid on amino-pentosides was determined on one hand by the temperature, and on the other hand on the duration of the reaction. The optimum time and temperature vary for every individual sugar, and have to be determined empirically.

When the optimum conditions were discovered the yield of xylohexosaminic acid rose from 10 per cent to 50 per cent. True, the reaction is so sensitive that often in spite of the best care the optimum conditions are missed and the yield falls from 50 to 30 or 25 per cent of the employed amino-pentoside.

Chondrosaminic and Epichondrosaminic Acids.

The conditions of the synthesis of lyxohexosaminic acid are the following. 60 gm. of amino-lyxoside dried first under diminished pressure over soda lime and subsequently in a vacuum desiccator at 50°C. were taken up in 80 cc. of water. To the suspension 36 cc. of an 80 per cent solution of prussic acid in water and 5 cc. of ammonia water were added. The flask, provided with a thermometer, was immersed in a warm water bath and the temperature maintained between 37–40°C. As soon as the temperature showed a slight tendency to rise the flask was immediately transferred to a cold water bath until the temperature fell to 37°C. The amino-pentoside rapidly dissolved, giving rise to a slightly brown solution which gradually turned dark and quite viscous. After a certain depth

[4] Levene and La Forge, *J. Biol. Chem.*, 1915, xxi, 351; xxii, 331.

of color and a certain viscosity are obtained, which one learns to recognize by experience, the reaction is interrupted. Generally it requires about 15 minutes to complete the reaction. The flask is then immersed into a cooling mixture and the temperature of the solution is allowed to fall to 0°C., and is poured into 300 cc. of concentrated hydrochloric acid previously cooled to the same temperature. The solution is then saturated with hydrochloric acid gas and allowed to stand not less than 1 hour; it may be allowed to stand over night. The solution is concentrated under diminished pressure at a temperature of the water bath not exceeding 75°C. In course of the concentration considerable ammonium chloride crystallizes out in the flask. When the solution becomes quite viscous the concentration is interrupted and the contents of the flask are poured into 2 liters of 95 per cent alcohol. Generally a small quantity of tar separates out which is removed by filtration through cotton wool. The alcoholic filtrate is concentrated under diminished pressure, the residue is dissolved in about 1 liter of water, and 200 gm. of moist barium hydroxide are added and the mixture is repeatedly evaporated to nearly dryness, the water being renewed at this phase. When all ammonia is removed the evaporation is interrupted and the barium is removed quantitatively by means of sulfuric acid, the hydrochloric acid by means of lead and silver carbonate. The final solution is concentrated under diminished pressure to a volume of about 50 cc. To this solution hot methyl alcohol is added gradually in small portions, avoiding an excess which causes precipitation of gum. On scratching, the α-aminohexonic acids begin to crystallize immediately and crystallization is completed in 24 hours.

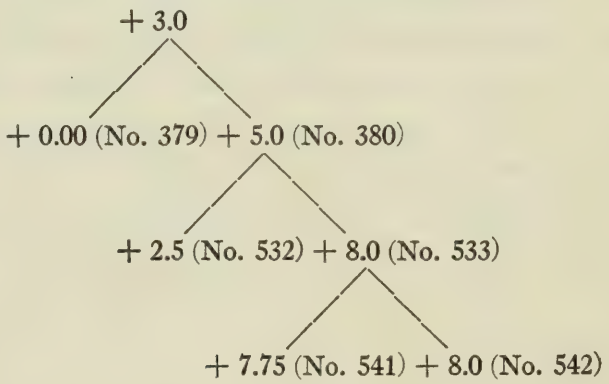
The specific rotation of the crude material was $[\alpha]_D^{20} = +3.0.^\circ$ Since the specific rotation of chondrosaminic acid is $[\alpha]_D^{20} = -17.0^\circ$ it is evident that the material is a mixture of the two epimers.

Separation of the Epimers.

250 gm. of mixed acid having $[\alpha]_D^{20} = +3.0^\circ$ were dissolved in 750 cc. of hot water and allowed to stand over night at room temperature. By far the greater part of the material crystallized out. The rotation of this substance (No. 379) was $[\alpha]_D^{20} = 0.00^\circ$. The mother

liquor was concentrated under diminished pressure to dryness and the residue again crystallized out of a minimum amount of water. A crystallime deposit formed on standing over night (No. 532) with rotation $[\alpha]_D^{20} = + 2.5°$. The mother liquor was concentrated to dryness, taken up in a minimum amount of water, and hot methyl alcohol was added to opalescence. Soon a crystalline deposit formed (No. 533) which had the rotation $[\alpha]_D^{20} = + 8.0°$. This material was again dissolved in a minimum amount of hot water and allowed to stand at room temperature over night. A crystalline deposit formed (No. 541) with the rotation of $[\alpha]_D^{20} = + 7.75°$. The mother liquor was concentrated under diminished pressure to dryness. The residue was taken up in a minimum amount of water and hot methyl alcohol was added until the appearance of a slight opalescence; the solution soon turned into a crystalline mass. The substance had the rotation $[\alpha]_D^{20} = + 8°$, thus showing that the final treatment did not accomplish any further fractionation.

The progress of fractionation is represented in the following diagram.



The substance with the rotation $[\alpha]_D^{20} = 0.00°$ was continually recrystallized out of water until a fraction was obtained with a rotation $[\alpha]_D^{20} = - 17.5°$.

The dextro epimer had the following composition.

0.1004 gm. substance gave 0.1358 gm. CO_2 and 0.0624 gm. H_2O.

	Calculated for $C_6H_{13}NO_6$: *per cent*	Found: *per cent*
C	36.92	36.89
H	6.66	6.96

The melting point was 206° (uncorrected) with gas evolution. The optical rotation was as follows:

$$[\alpha]_D^{20} = \frac{+0.16 \times 100}{1 \times 2} = +8°$$

The rotation of chondrosaminic acid being − 17.0° and that of the epimer + 8.0°, the numerical value of the α-carbon atom is $\frac{8 - (-17.0)}{2} = 12.5°$.

Xylohexosaminic Acids.

Xylohexosaminic acid, as prepared previously by Levene and La Forge, consisted of a mixture of the two epimers, in which the dextro acid was in preponderance. At that time, this was not realized, and the substance was considered the pure dextro acid. Since some theoretical deductions were based on the earlier conception, it became necessary to repeat the earlier work on the pure epimer.

Synthesis of d-Xylohexosaminic Acids.

The general plan of the preparation of the material is as out lined in the paragraph on the preparation of lyxohexosaminic acids The points of difference were the temperature of the reaction and the duration of the interaction of prussic acid and the amino-pentoside. The temperature was not allowed to exceed 35°C., and the experiment was continued for 30 minutes. The further treatment was identical with that for the preparation of lyxohexosaminic acids.

Separation of the Epimers.—280 gm. of the mixed acids were dissolved in a minimum amount of water and methyl alcohol was added until a crystalline precipitate began to form. This was filtered off. It had a rotation of $[\alpha]_D^{20} = +5.0°$. The mother liquor was concentrated to dryness under diminished pressure. The residue was then taken up in a minimum amount of water and on addition of alcohol a precipitate formed, which was removed by filtration and had the rotation $[\alpha]_D^{20} = +2.00°$ (No. 615).

180 gm. of this substance were dissolved in 300 cc. of hot water, and the solution was allowed to cool. A precipitate (No. 630) thus formed had a rotation $[\alpha]_D^{20} = -1.0°$. 110.0 gm. of No. 630

were dissolved in 400 cc. of hot water and the solution was allowed to stand over night and a crystalline deposit (No. 641) formed having a rotation $[\alpha]_D^{20} = -1.0°$. On further fractionation of No. 641 the rotation of the substance did not change. The substance melted at 200°C. (uncorrected) with gas evolution. The composition was the following.

0.1000 gm. substance gave 0.1354 gm. CO_2 and 0.0590 gm. H_2O.
0.1000 " " required for neutralization 5.25 cc. of N acid.

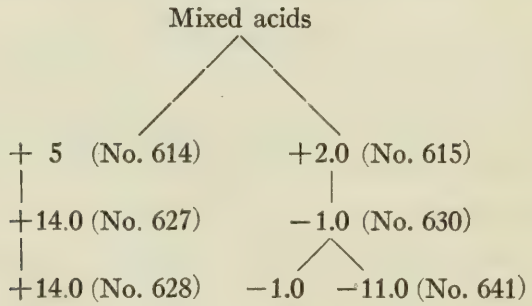
	Calculated for $C_6H_{13}NO_5$: *per cent*	Found: *per cent*
C	36.92	36.92
H	6.66	6.60
N	7.18	7.35

The rotation of the substance was:

Initial.
$$[\alpha]_D^{20} = \frac{-0.22 \times 100}{1 \times 2} = -11.0°$$

Equilibrium.
$$[\alpha]_D^{20} = \frac{-0.63 \times 100}{1 \times 2} = -31.5°$$

The progress of fractionation of the epimers is as follows:



The substance with rotation $[\alpha]_D^{20} = +5°$ was dissolved in a minimum amount of boiling water, allowed to stand 30 minutes, and while still warm was filtered. The precipitate thus formed had the rotation $[\alpha]_D^{20} = +14.0°$. The yield of the substance was 87 gm. These were dissolved in 400 cc. of boiling water. On cooling, these formed a precipitate which was removed by filtration. The mother liquor was concentrated to dryness under diminished pressure. The residue was taken up in a minimum amount of water and hot methyl alcohol was added to opalescence. The crystallization began immediately. The optical rotation of both substances was identical: $[\alpha]_D^{20} = +14.0°$.

Thus the optical rotation of the two epimers obtained by fractional crystallization was + 14.0° and − 11.0° respectively, and the rotation of the α-carbon atom is therefore $\frac{A - B}{2} = \frac{14 + 11}{2} = 12.5°$. The purity of the two epimers was ascertained by the conversion of the dextroisomer into its lactone hydrochloride, and the conversion of the levoepimer into the benzal lactone hydrochloride. Both lactones were subsequently converted into their parent acids.

d-Dextroxylohexosaminic Lactone Hydrochloride.

The lactone was prepared in the manner described in the following publication.[5] It was recrystallized from an excess of methyl alcohol. The substance had the melting point of 205°C. (uncorrected) with gas evolution. The substance had the following composition.

0.1000 gm. substance gave 0.1234 gm. CO_2 and 0.0504 gm. H_2O.
0.2000 " " required for neutralization 9.31 cc. 0.1 N acid.
0.1000 " " " " titration of HCl 4.5 cc. N $AgNO_3$.

	Calculated for $C_6H_{12}NO_5Cl$: *per cent*	Found: *per cent*
C	33.71	33.65
H	5.67	5.64
N	6.55	6.52
Cl	16.25	15.98

Conversion of the Lactone Hydrochloride into the Parent Acid.— 5 gm. of the lactone were dissolved in 50 cc. of water. An excess of barium hydroxide was added, and the solution was allowed to stand over night. The barium and the chlorine were then removed quantitatively. The filtrate was concentrated, and the acid crystallized on the addition of hot methyl alcohol. The substance had the melting point of 224° (uncorrected) with gas evolution. The substance had the following composition.

0.1070 gm. substance gave 0.1372 gm. CO_2 and 0.0620 gm. H_2O.
0.0100 " " " 1.27 cc. nitrogen in the Van Slyke micro-apparatus at 26°C. and 759 mm. pressure.

	Calculated for $C_6H_{13}NO_5$: *per cent*	Found: *per cent*
C	36.92	37.04
H	6.66	6.86
N	7.18	6.99

[5] Levene, *J. Biol. Chem.*, 1918, xxxvi, 89.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{+0.28 \times 100}{1 \times 2} = +14.0°$$

Monobenzal d-Levoxylohexosaminic Lactone Hydrochloride.

2.5 gm. of the *d*-levohexosaminic acid were taken up in a solution of 15 cc. of 99.5 per cent of alcohol and 3 cc. of benzaldehyde. Hydrochloric acid gas was passed through the solution. The amino-acid went gradually into solution and after some time the entire solution solidified instantaneously. After 1 hour 2 cc. of ether (dried over sodium) were added and the material was allowed to stand an additional 2 hours. The lactone was then filtered and recrystallized out of methyl alcohol. The melting point was 206°C. (uncorrected) with gas evolution.

0.1023 gm. substance gave 0.1938 gm. CO_2 and 0.0508 gm. H_2O.
0.1000 " " required for neutralization 3.34 N acid.

	Calculated for $C_{13}H_{15}NO_5$. HCl (molecular wt. 301.6)	Found:
C	51.72	51.81
H	5.35	5.57
N	4.69	4.68

The optical rotation of the substance in 50 per cent alcohol was:

$$[\alpha]_D^{20} = \frac{-1.21 \times 100}{1 \times 2} = -60.5°$$

Conversion of the Lactone into the Free Acid.—10 gm. of the dry monobenzal lactone hydrochloride were dissolved in 50 cc. of water, and to the solution were added 12.5 gm. of barium hydroxide containing 5.0 gm. of Ba. The solution was placed on a boiling water bath. It was soon noticed that on warming the solution turned yellow and developed ammonia. The experiment was therefore interrupted after 7 minutes. The solution was then rendered acid with sulfuric acid. The filtrate from the barium sulfate precipitate was extracted repeatedly with ether, and the aqueous solution was freed quantitatively from sulfuric and hydrochloric acids. The filtrate concentrated and the amino-acid crystallized on addition of hot methyl alcohol. The melting point of the acid was 200°C. (uncor-

rected) with gas evolution. The substance had the following composition.

0.1000 gm. substance gave 0.1354 gm. CO_2 and 0.600 gm. H_2O.
0.0100 " " in the Van Slyke micro-apparatus gave 1.36 cc. nitrogen at 35°C. and 762 mm. pressure.

	Calculated for $C_6H_{13}NO_6$:	Found:
C	36.92	36.98
H	6.66	6.71
N	7.18	7.16

The optical rotation of the substance was:

Initial. $[\alpha]_D^{20} = \frac{-0.22 \times 100}{1 \times 2} = -11.0°$

Equilibrium. $[\alpha]_D^{20} = \frac{-0.60 \times 100}{1 \times 2} = -30.0°$

Thus the rotations of the epimers obtained from the lactones were $+14.08°$ and $-11.0°$ respectively, and the rotation of their α-carbon atoms is $\frac{A - B}{2} = \frac{14 + 11}{2} = 12.5°$.

[Reprinted from The Journal of Biological Chemistry, October, 1918, Vol. xxxvi, No. 1, pp. 89–94.]

THE ACTION OF NITROUS ACID ON EPIMERIC HEXOSAMINIC ACIDS.

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 30, 1918.)

It was definitely proven by the observation of Levene and La Forge,[1] that in course of the reaction of nitrous acid on chitosamine and on chitosaminic acid respectively, a Walden inversion takes place in one of the two substances. It was also shown[2] that under suitable conditions by the action of nitrous acid on the benzal of chitosaminic ethyl ester a diazo derivative is formed as an intermediary phase. Similarly, it was found by Levene and LaForge that chondrosamine and chondrosaminic acid, likewise xylohexosaminic acid and its lactone, gave rise to epimeric acids.

Accepting for the structure of the diazo compound the expression

$$CH_2(OH)(CHOH)_3 - C(N=N) - COON$$

it becomes evident that each one of a pair of epimers should on the basis of conventional theories lead to one and the same diazo derivative, and hence to one and the same deaminized acid. On the other hand, if two epimeric amino-acids, passing through the phase of the diazo derivative, give rise to two epimeric deaminized acids, one would be forced to accept the existence of two isomeric diazo bodies, perhaps of the nature of electromers. In view of these considerations it became important to obtain possession of epimeric amino-acids. Three such pairs have been prepared as reported in the preceding communication.[3] They are chitosaminic and epichitosaminic,

[1] Levene, P. A., and LaForge, F. B., *J. Biol. Chem.*, 1915, xxi, 351.

[2] Levene and LaForge, *J. Biol. Chem.*, 1915, xxi, 345.

[3] Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 73.

chondrosaminic and epichondrosaminic, *d*-dextroxylohexosaminic and *d*-levoxylohexosaminic acids. In each one of the three pairs the epimeric amino-acids gave rise to epimeric anhydro sugar acid as may be seen from the following tabulation.

A.	Chitosaminic	anhydro-*d*-gluconic	acid.
	Epichitosaminic	" -*d*-mannonic	"
B.	Chondrosaminic	" -*d*-mucic	"
	Epichondrosaminic	" -*d*-talomucic	"
C.	*d*-Dextroxylohexosaminic	" -*d*-idosaccharic	"
	d-Levoxylohexosaminic	" -*l*-saccharic	"

One peculiarity was observed in course of the work; namely, whereas dextroxylohexosaminic acid and its lactone gave anhydro-*d*-idosaccharic and anhydro-*l*-saccharic acid respectively, the epichitosaminic acid and its lactone formed the same anhydro-*d*-mannonic acid. Thus, while in the first instance a Walden inversion took place, in either the acid or the lactone, in the second, the inversion occurred in neither or in both. Similar irregularities were observed by Fischer and his coworkers in their study of the action of nitrous acid on amino-acids and their esters.

Whether the lactone and the corresponding hexosaminic yield the same deamino-acid could not as yet be established. Epichondrosamine has been obtained synthetically, but the sugar was found to possess such peculiarities that up to the present the attempt to convert it either into the amino-acid or into the deaminized acid has failed. The preparation of larger quantities of the sugar are now in progress.

EXPERIMENTAL.

The deamination of the amino-acids was brought about in the same manner for all acids. 5 gm. of the acid were dissolved in 50 cc. of 7.5 per cent hydrochloric acid. To the solution 5 gm. of silver nitrite were added. The flask containing the mixture was immersed in an ice and alcohol cooling mixture. The bath was allowed to warm up gradually to room temperature. After 12 to 16 hours a new portion of 2 gm. of silver nitrite and 2 cc. of 10 per cent hydrochloric acid was added. The material was then

allowed to stand 4 to 6 hours at room temperature. The resulting solution was freed from silver by means of hydrogen sulfide, the final filtrate concentrated, under diminished pressure, to a small volume and further treated according to the aim of the individual experiment.

Lactones were deaminized in a similar manner, the variation consisting in that 5 gm. of the lactone hydrochloride were dissolved in 50 cc. of distilled water, to which about 1 cc. of 10 per cent hydrochloric acid was added.

Deamination of Epichitosaminic Acid.

The mother liquor after deamination was reduced by means of aluminum amalgam according to the method of Levene and Meyer,[4] and the resulting solution neutralized with calcium carbonate, and concentrated under diminished pressure to a small volume. The composition of the Ca salt was the following.

0.1020 gm. substance gave 0.1244 gm. CO_2, 0.0482 gm. H_2O, and 0.0134 gm. CaO.

	Calculated for $(C_6H_9O_6)_2Ca+2H_2O$:	Found:
C	33.49	33.26
H	5.12	5.07
Ca	9.30	9.38

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.71 \times 100}{1 \times 2} = +35.5°$$

Under the same conditions the lactone gave a salt of the following composition.

0.1030 gm. substance gave 0.1282 gm. CO_2, 0.0484 gm. H_2O, and 0.0138 gm. CaO.

	Calculated for $(C_6H_9O_6)_2Ca+2H_2O$:	Found:
C	33.49	33.94
H	5.12	5.26
N	9.30	9.56

[4] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 599.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.71 \times 100}{1 \times 2} = +35.5°$$

On deamination of chitosaminic acid chitaric acid is formed.

Deamination of Epichondrosaminic Acid.

In this instance the dicarboxylic acid possesses more convenient properties, and hence the product of deamination was oxidized further by means of nitric acid. For this purpose the solution obtained on deamination of 6 gm. of the amino-acid was concentrated to 35 cc., and an equal volume of concentrated nitric acid was added, the solution heated over a flame until the evolution of red fumes, and then the heating maintained for 7 minutes. The reaction product was rapidly evaporated with constant stirring on a clock glass placed on a boiling water bath. The residue was redissolved in a solution of 5 cc. of water and 5 cc. of nitric acid and again evaporated to dryness. The product was then evaporated twice with water in order to remove the adhering nitric acid. Finally the aqueous solution was converted into the calcium salt. The yield was 3.5 gm. of the crude calcium salt. The crude material was purified by removing the calcium by means of oxalic acid and reconverting the filtrate into the calcium salt. The dry substance had the following composition.

0.0988 gm. substance gave 0.1042 gm. CO_2, 0.0302 gm. H_2O, and 0.0222 gm. CaO.

	Calculated for ($C_6H_8O_8Ca$):	Found:
C	29.03	28.76
H	3.22	3.44
CaO	22.58	22.47

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.45 \times 100}{1 \times 5} = -9.0°$$

Under the same conditions of oxidation chondrosaminic acid yields the inactive anhydromucic acid.

Deamination of d-Dextroxylohexosaminic Acid.

In the earlier work of Levene and LaForge it was found that xylohexosaminic acid and its lactone gave rise to *d*-anhydroidosaccharic and *l*-anhydrosaccharic acid. Since this work was carried out on a material which consisted of a mixture of the two epimers, it became necessary to verify these earlier experiments.

10 gm. of pure *d*-dextroxylohexosaminic acid were converted into anhydroidosaccharic acid in the same manner as previously described by Levene and LaForge. This time the substance crystallized after the first evaporation. It was taken up in a minimum amount of water and several volumes of acetone were added. On standing, the substance crystallized. The yield of recrystallized substance was 4.8 gm. The composition of the substance was the following.

0.1128 gm. substance lost on weighing 0.0186 gm.

	Calculated for $C_6H_8O_7+2H_2O$:	Found:
H_2O	15.80	16.49

0.0942 gm. dry substance gave on combustion 0.1282 gm. CO_2 and 0.0362 gm. H_2O.

	Calculated for $C_6H_8O_7$:	Found:
C	37.50	37.11
H	4.20	4.30

The rotation of the substance was $[\alpha]_D^{20} = \frac{-1.56 \times 100}{1 \times 2} = -78.0°$, or calculated for the dry substance $= -93.4°$.

On oxidation of the lactone, the acid potassium salt of anhydrosaccharic acid was obtained.

Deamination of d-Levoxylohexosaminic Acid.

7 gm. of the acid were deaminized in the way described above. The reaction product was brought, by distillation under diminished pressure, to a volume of 30 cc. An equal volume of nitric acid was added. The solution was heated over a free flame until the beginning of the evolution of fumes; it was then gently heated for 7 minutes after which the solution was transferred to a clock glass and evaporated on a water bath to dryness. The residue was again

dissolved in a solution consisting of 5 cc. of nitric acid and 5 cc. of water. The final product was converted into the acid potassium salt in the manner described by Levene and LaForge. The yield of the crude product was 3.05 gm. The salt was recrystallized three times and then had the following composition.

0.1000 gm. substance gave 0.0366 K_2SO_4.

	Calculated for $C_6H_7O_7K+H_2O$:	Found:
K.....	15.70	16.03

The optical rotation was

$$[\alpha]_D^{20} = \frac{-0.75 \times 100}{1 \times 2} = -37.50°$$

[Reprinted from The Journal of Biological Chemistry, October, 1918, Vol. xxxvi, No. 1, pp. 105–126.]

MUCINS AND MUCOIDS.

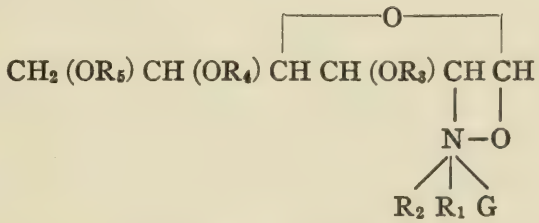
By P. A. LEVENE and J. LÓPEZ-SUÁREZ.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 23, 1918.)

The structure of mucin and allied substances has remained in obscurity until recent years, although these substances interested both the chemist and the physiologist. Knowledge was lacking, particularly regarding the mode of union between the sugar and the rest of the molecule. The information given in text-books was generally limited to that of the solubilities and some other physical properties. The substances were classified in three groups: mucins, mucoids, and glucoproteins.

More recently Irvine and Hynd[1] had suggested the following structure of this group of substances.



According to this conception the amino group of the nitrogenous sugars serves as a bridge for the linking of amino-acids to the sugar, in a peptide as well as in a glycosidic union. The hypothesis suggested itself to Irvine and Hynd on the ground of their work on the glycosides of chitosamine, and was based entirely on speculative evidence. Irvine expressed the view rather tentatively, realizing the need of experimental proof.

On the other hand, evidence was accumulating tending to show that all substances allied to mucins contained in their molecule a

[1] Irvine, J. C., and Hynd, A., *J. Chem. Soc.*, 1913, ciii, 41.

complex acid which in its structure bore no similarity or relationship to peptides.

For many years it was known that an acid of the type of conjugated sulfuric acid existed in the molecule of chondromucoid.[2] Later its existence was revealed also in tendomucoid. The acid under the name of chondroitin sulfuric acid was discovered by Mörner, and received further attention through the efforts of Schmiedeberg at a time when methods applied to the study of carbohydrates were very imperfect. Later, in course of the work it was found that the acid could not be isolated with equal facility from every mucin or mucoid.[3,4] In many instances the removal of the protein radicle was accomplished only after numerous operations, and in course of these operations the conjugated sulfuric acid suffered partial decomposition. It was not clear (and it partly still remains so) whether the differences of individual mucins lay in the protein or in the acid radicles.

Finally a chemical distinction was discovered between the conjugated sulfuric acids of different origin.[5] The difference was in the components. Whereas one contained in its molecule the nitrogenous hexose, chondrosamine, the other had in its place chitosamine. For the former the name chondroitin sulfuric acid was retained, the latter was named mucoitin sulfuric acid.

The substances of the first group revealed a comparatively greater resistance towards hydrolytic agents and could be obtained in a fair degree of purity, whereas the substances of the second group when prepared free from protein always appeared in form of a mixture of mucoitin sulfuric acid with mucoitin. Also the intermediary substances showed a difference in their resistance towards hydrolytic agents, so that chondrosin could be prepared in a practically pure state whereas mucosin was found so labile that, under the conditions which permitted the preparation of the former, the latter was nearly completely hydrolyzed. On the other hand, when mucoitin sulfuric

[2] Mörner, C. T., *Skand. Arch. Physiol.*, 1889, i, 210.

[3] Levene, P. A., *Z. physiol. Chem.*, 1900–01, xxxi, 395.

[4] Mandel, J. A., and Levene, P. A., *Z. physiol. Chem.*, 1905, xlv, 386. López-Suárez, J., *Biochem. Z.*, 1913, lvi, 167. Alzona, F., *ibid.*, 1914, lxvi, 408.

[5] Levene, P A., and López-Suárez, J., *J. Biol. Chem.*, 1916, xxv, 511; xxvi, 373.

acid was subject to milder hydrolysis a mucosin resulted which still contained undecomposed mucoitin.

Because of this instability of mucoitin sulfuric acid, the acid itself and the products of its partial hydrolysis were not obtained in the same degree of purity as chondroitin sulfuric acid and the corresponding products derived from it.

Furthermore, in course of the present work some differences were observed between various members of the mucoitin sulfuric acid group. It is true that, since the individual members were not always obtained in absolutely pure state, there is present also the possibility that these differences were brought about by impurities. However, the impression still remains that the acids of this group belong to two types. A representative of one type is the substance derived from funis mucin. The acid of this type is characterized by its gelatinous nature when precipitated by glacial acetic acid, by the comparatively small quantity of glacial acetic acid required for its precipitation, and by the readiness with which it forms an insoluble barium salt. The barium salt is very sparingly soluble in water, but is readily dissolved in the presence of acetates.

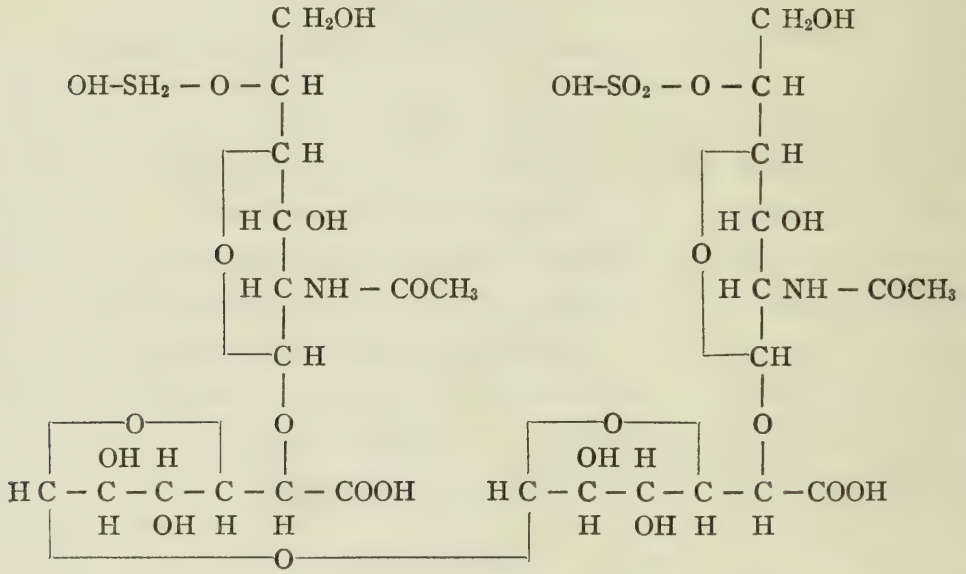
The second type of mucoitin sulfuric acid is characterized by the greater solubility of the barium salt, by the fact that the substance is precipitated by glacial acetic acid in form of a flocculent precipitate, and by the fact that a large excess of the acid is required in order to bring about the precipitation. A representative member of this type is the substance obtained from gastric mucus.

Structure of Mucoitin Sulfuric Acid.

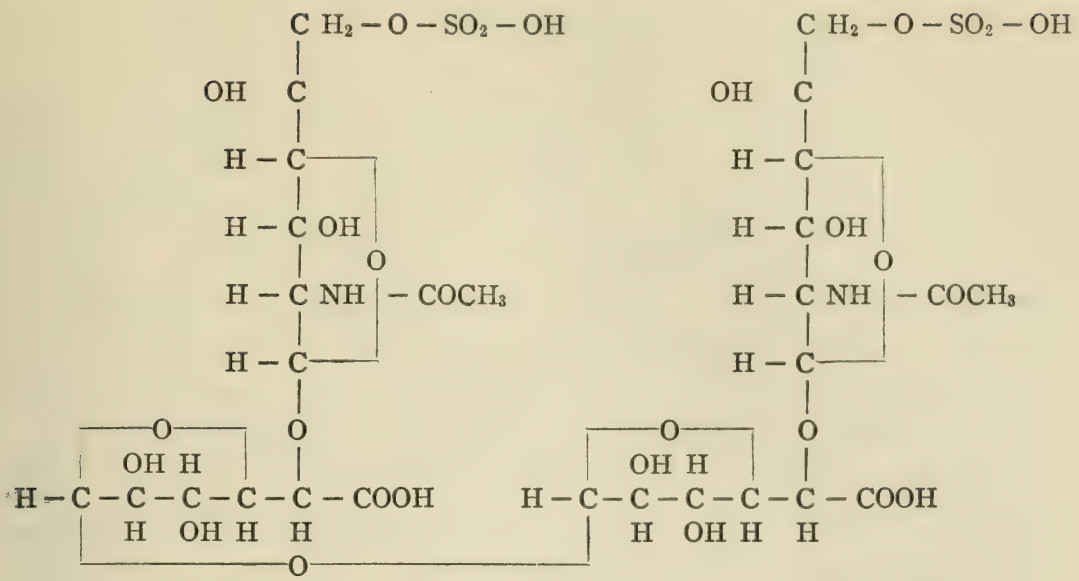
Concerning the structure of the acids of this group, it seems permissible to assume the same hypothesis as was previously formulated by Levene and La Forge for chondroitin sulfuric acid. On removal of sulfuric acid a substance is formed which is analogous to chondroitin. It is non-reducing, and does not contain free amino groups. On hydrolysis it forms mucosin, which is a disaccharide composed of glucuronic acid and chitosamine. Mucoitin, similarly to chondroitin, contains one acetyl group to each molecule of chitosamine, glucuronic, and sulfuric acids.

The glucuronic acid was identified in all the acids of the mucoitin sulfuric acid group but not with the same degree of exactness. Whereas in the acids of the funis mucin type the glucuronic acid was identified by the formation of furfural, by the analysis of the phenylhydrazine derivatives, and by the analysis of the acid potassium salt of saccharic acid formed on oxidation with nitric acid; in the acids of the second group it was demonstrated only by the furfural distillation, and by the formation of the phenylhydrazine, which, however, was obtained in a quantity too small for purification and analysis.

As regards the details of the structure all the arguments which suggested the structural formula of chondroitin sulfuric acid may be repeated for the suggestion of a graphic formula for mucoitin sulfuric acid. This is as follows:



On this occasion the structure of chondroitin sulfuric acid is presented for comparison though in a form slightly different from that originally suggested by Levene and La Forge. The difference is introduced in that part which expresses the structure of the amino sugar. In the early period of the work the amino-hexose was assumed to be derived from *l*-ribose. It was later established that the configuration of the sugar was that of a *d*-lyxohexosamine. The substance is graphically presented as follows:



In both formulas the position of the sulfuric acid radicle, the place of union of sugar and glucuronic acid, and the position of the amino groups are arbitrary. To the sulfuric acid is assigned a different position in the two substances in order to indicate the existence of a difference in their respective behavior towards hydrolytic agents.

Distribution of the Acids of Various Types.

The distribution of the acids in various organs and tissues was found to be the following:

I. Chondroitin sulfuric acid.
- 1 Cartilage.
- 2 Tendons.
- 3 Aorta.
- 4 Sclera.

II. Mucoitin sulfuric acid.

A.
- 1 Funis mucin.
- 2 Humor vitreous.
- 3 Cornea.

B.
- 1 Mucin of gastric mucosa.
- 2 Serum mucoid.
- 3 Ovomucoid.
- 4 Ovarian cysts.

Table I contains a summary of the analytical data obtained on the individual substances.

TABLE I.

		Theory.	Group I.				Group II.				
							A.			B.	
			Cartilage.	Tendons.	Aorta.	Sclera.	Navel cord.	Vitreous humor.	Cornea.	Gastric mucus.	Serum mucoid.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
	C	27.80		25.13		34.27	33.39	34.39	37.83		
	H	3.48		9.88		4.86	5.38	5.72	6.22		
	N	2.32	2.14	2.11	2.54	5.66	4.03	4.96	4.62	3.47	5.25
$C_{26}H_{44}O_{29}N_2S_2Ba_2$.	S	5.30	3.72	4.26	2.40	4.57	4.07	3.63	3.10	1.48	1.96
Mucoitin, chondroitin, sulfuric acid.	Base.	22.70		18.35		28.83		21.50	14.74		29.14
	Furfural.	33.0					17.5	23.0		13.0	
	Acetyl.	10.0					9.39			7.8	
	$[\alpha]_D$		−25.0				−45.6	−49.2		−22.54	
Glucuronic acid derivatives.			+	+	−	−	+			+	
	C	36.9	37.3	35.8			38.25				
	H	5.64	5.66	6.0			5.80				
$C_{12}H_{21}O_{11}N.HCl$.	N	3.58	3.45	3.45	4.34		−				
Chondrosin or mucosin.	NH_2-N	3.58	3.45	3.45	3.41		3.24			2.12	
	$[\alpha]_D$		+43.4	+41.5	+42.0		+25.55			+25.75	
Sugar.			Chondrosamine.				Glucosamine.				

EXPERIMENTAL.

Group I.

Aorta Mucoid.

Preparation of Chondroitin Sulfuric Acid.

100 pounds of aorta freed from extraneous tissue were put through a hashing machine, taken up in 20 liters of a 2 per cent solution of sodium hydroxide, and allowed to stand for 36 hours. The extract was decanted and the residue again extracted for another 36 hours. The combined solutions were strained, neutralized, concentrated with an excess of barium carbonate, filtered, and finally precipitated with glacial acetic acid. The precipitate had the appearance of chondroitin sulfuric acid. The precipitate was washed with glacial acetic acid, then with alcohol, and dried. The yield was 40.0 gm. Of these 8 gm. were used for hydrolysis and the remaining material was purified in the following manner.

The material was again dissolved in water and precipitated with glacial acetic acid. The precipitate was washed with glacial acetic acid and then with alcohol. The dry precipitate was dissolved with the aid of potassium hydroxide. To this solution a slight excess over the required amount of barium chloride was added, followed by the addition of an equal volume of 95 per cent alcohol. The precipitate was washed by decantation with 50 per cent alcohol until free from barium chloride. The washing was then continued with alcohol of progressively increasing strength, finally with ether, and dried. The yield of this material was 25 gm.

Analysis of Substance.

0.200 gm. substance required for neutralization 3.63 cc. 0.1 N acid.
0.200 " " gave on fusion 0.0354 gm. $BaSO_4$.
0.0778 " " " " combustion 0.0818 gm. CO_2 and 0.0386 gm. H_2O.

	Calculated for $C_{26}H_{44}O_{29}N_2S_2Ba_2$:	Found:
C	27.8	28.7
H	3.48	3.35
N	2.32	2.54
S	5.30	2.4

Hydrolysis of Substance for Identification of Sugar.

8 gm. of the substance with 60 cc. of 20 per cent hydrochloric acid, together with 1.5 gm. of barium chloride and 1.5 gm. of stannous chloride, were hydrolyzed with reflux condenser for 12 hours. The product of hydrolysis was freed from barium and tin and the filtrate concentrated under diminished pressure, warmed to about 50°C. in a water bath. On concentration the sugar crystallized in long, microscopic, prismatic needles. These were transferred to a flask by means of alcohol containing hydrochloric acid. The flask was allowed to stand over night and the precipitate was then filtered and washed with alcohol and ether. The yield was about 1.0 gm. The melting point was 183° (uncorrected).

0.020 gm. gave in the Van Slyke apparatus 2.30 cc. of nitrogen gas at 25°C. and 762.7 mm. pressure.

	Calculated for $C_6H_{13}O_5N$. HCl:	Found:
N	6.51	6.40

The rotation of the substance was the following:

Initial.

$$[\alpha]_D^{25} = \frac{+1.64 \times 2.0593}{1 \times 0.0508} = +65.6°$$

Equilibrium.

$$\frac{+2.27 \times 2.0593}{1 \times 0.0508} = +91.15°$$

Preparation of Chondrosin.—20 gm. of chondroitin sulfuric acid were hydrolyzed for 1 hour in a boiling water bath with 60 cc. of 20 per cent hydrochloric acid. The reaction product was filtered, concentrated under diminished pressure, and precipitated by means of alcohol and ether. The yield was 4 gm. The analysis of the substance gave the following results.

0.100 gm. required for neutralization 3.10 cc. 0.1 N acid.

0.020 gm. gave in the Van Slyke micro-apparatus 1.2 cc. N at 25°C. and 759.6 mm. pressure.

	Calculated for $C_{12}H_{21}O_{11}N$. HCl:	Found:
N	3.58	4.34
NH_2–N	3.58	3.41

The rotation of the substance was the following:

$$[\alpha]_D^{25} = \frac{+1.04 \times 2.0026}{1 \times 0.0496} = +42.00°$$

Sclera Mucoid.

Preparation of Chondroitin Sulfuric Acid.

For the preparation of this substance, originally the sclera and cornea were worked up in one. The hydrolysis of the substance, however, revealed the presence of two sugars, chitosamine and chondrosamine. Because of this in later experiments the cornea was dissected out. The sample used for ultimate analysis was prepared from sclera and cornea combined. But the sugar fraction still contained some glucosamine. On the other hand the mucoid from the cornea contained some glucosamine. The sclera was the only tissue which yielded mucoid containing both sugars, and the possibility is not excluded that one of them (chitosamine) is derived from adhering extraneous tissues.

The procedure for the preparation of the conjugated sulfuric acid was the following. The eyes were freed from adhering muscle and connective tissue, then the humor vitreous, lens, and retina were removed. Finally, the cornea and sclera were washed with running water from all extraneous material. After this the corneas were carefully dissected out, and the scleras were minced in the hashing machine, and placed in a large volume of a 3 per cent sodium hydroxide. For the corneas of 1,000 eyes 20 liters of sodium hydroxide were used. The extraction was continued 3 days, at the end of which time the solution was strained through cheese-cloth and neutralized with acetic acid. Barium carbonate was then added in excess and the mixture was concentrated on a water bath to a small volume. The product of the reaction was filtered on a suction funnel and then converted into the lead salt. The lead salt was treated in the usual way. It was converted into the barium salt for the analysis of conjugated sulfuric acid. For the isolation of the sugar it was hydrolyzed directly.

Analysis of the Barium Salt.

0.1903 gm. substance required for neutralization 7.69 cc. 0.1 N acid.
0.2855 " " gave 0.0950 gm. $BaSO_4$.
0.0986 " " " 0.1239 gm. CO_2 and 0.0428 gm. H_2O.

	Calculated for $C_{26}H_{44}O_{29}N_2S_2Ba_2$:	Found:
C	27.8	34.27
H	3.48	4.86
N	2.32	5.66
S	5.30	4.57
Ba	22.70	23.83

Isolation of the Sugar.—12.0 gm. of the lead salt with 2 gm. stannous chloride, 2 gm. of barium chloride, and 60 gm. of 20 per cent hydrochloric acid were heated with reflux condenser 10 hours over free flame. The reaction product was freed from lead, tin, and barium, and then concentrated to syrup. This was dissolved in a minimum amount of hot methyl alcohol. Soon crystals appeared which had the typical appearance of chitosamine. These were filtered off and the mother liquor allowed to stand, from time to time a few drops of ether were added. At the end of a week the maximum amount of chondrosamine hydrochloride settled out. The best yield for 12 gm. was 0.5 gm. of the sugar.

The melting point of this was 182° (corrected). The analysis of the substance was as follows:

0.020 gm. substance gave 2.39 cc. of nitrogen gas at 19°C. and 745 mm. pressure.

0.0523 gm. substance required for titration of the hydrochloric acid 2.39 cc. of $AgNO_3$ (1 cc. = 0.003546 gm.).

0.1036 gm. substance gave 0.1224 gm. CO_2 and 0.0602 gm. H_2O.

	Calculated for $C_6H_{13}O_5N.HCl$:	Found:
C	33.40	33.18
H	6.54	6.90
N	6.57	6.38
Cl	16.45	16.20

The rotation of the substance was the following:

$$[\alpha]_D^{25} = \frac{+1.42 \times 2.0408}{1 \times 0.0510} = +56.80° \quad \text{Initial.}$$

$$\frac{+2.39 \times 2.0408}{1 \times 0.0510} = +95.66° \quad \text{Equilibrium.}$$

Group II A.

Funis Mucin.

Preparation of Mucoitin Sulfuric Acid.

Larger quantities of the material were accessible this year. This fact permitted more careful purification. It was observed in the course of preparation of this substance that to obtain it could not be accomplished satisfactorily by means of lead acetate. It is essential to free the cords from all adhering blood clots and blood vessels, as otherwise the resulting substance is contaminated with nucleic acid. The separation of the two is very troublesome. The purification, however, was accomplished by vigorous treatment with glacial acetic acid. The treatment with lead acetate is a convenient step in order to free the substance from adhering salt as well as from other impurities.

In removing the lead by means of hydrogen sulfide one has to bear in mind the insolubility of the acid in water. Because of this, it is necessary to carry out the separation of lead in a slightly alkaline solution.

The details of the process as carried out at present are as follows: About 100 cords, freed from blood vessels, either shredded or chopped in a hashing machine, are taken up in 6 liters of 2 per cent of NaOH and allowed to stand 3 days, then acidulated and centrifugalized to remove the precipitate. The supernatant liquid was concentrated with an excess of barium carbonate on a water bath. This operation was continued 24 hours and the product centrifugalized. The supernatant liquid was allowed to stand on a hot water bath after a second addition of barium carbonate. Water was added from time to time. The operation was continued about 2 days. The resulting material was then centrifugalized, and the supernatant liquid allowed to stand until part of the barium acetate had crystallized out. The material was again centrifugalized and the clear supernatant solution precipitated with glacial acetic acid. The precipitate was redissolved in water on addition of barium acetate; the substance was reprecipitated out of this solution with glacial acetic acid. The crude material was washed with 95 per cent alcohol to remove the excess of

acetic acid. The material was dissolved in water, and the solution was neutralized with a solution of barium hydroxide until it reacted neutral to litmus.

To the final solution enough 95 per cent alcohol was added to precipitate the crude barium salt. This was washed first with 50 per cent alcohol to remove adhering barium acetate, then with alcohol of increasing concentration and finally with 99.5 per cent alcohol and with ether. The final product analyzed as follows:

0.1000 gm. substance required for neutralization 2.88 cc. 0.1 N acid.
0.2000 " " gave on fusion 0.0592 gm. $BaSO_4$.
0.0936 " " " " combustion 0.0450 gm. H_2O and 0.1146 gm. CO_2.

	Calculated for $C_{26}H_{44}O_{29}N_2S_2Ba_2$:	Found:
C	27.8	33.39
H	3.48	5.38
N	2.32	4.03
S	5.30	4.07

The optical rotation of the substance was as follows:

$$[\alpha]_D^{25} = \frac{-0.50 \times 5\ 0641}{1 \times 0.0555} = -45\ 6°$$

Preparation of Mucosin.—An attempt was made to prepare mucosin under the same conditions of hydrolysis as employed for preparation of chondrosin. However, the largest part of the substance underwent complete hydrolysis, with the formation of free chitosamine, which was identified in the usual way. It was found subsequently, that a substance analogous to chondrosin could be obtained under the following conditions.

4.5 gm. of the barium salt were dissolved in 100 cc. of 10 per cent hydrochloric acid and heated on a water bath for $\frac{1}{2}$ hour. The solution then contained all its nitrogen in form of uncombined amino nitrogen, and showed a reduction of Fehling's solution equivalent to 1.12 gm. of glucose; the theory requires 1.26 gm. The solution was freed quantitatively from barium, concentrated to 3 cc. under diminished pressure at a temperature of water bath not exceeding 45°. These were then gradually poured into 200 cc. of alcohol, to which 400 cc. of dry ether had been added. A white flocculent precipitate then formed. It was allowed to stand over night, then filtered, and dried. The yield was 1.5 gm.

0.0188 gm. substance gave in the Van Slyke micro-apparatus 1 cc. N at 27° and 759.3 mm. pressure.

0.0918 gm. substance gave 0.1288 gm. CO_2 and 0.0476 gm. H_2O.

	Calculated for $C_{12}H_{21}O_{11}N$. HCl:	Found:
C	36.9	38.25
H	5.64	5.8
NH_2-N	3.58	3.24

$$[\alpha]_D^{20} = \frac{+0.66 \times 2.0287}{1 \times 0.0524} = +25.55°$$

Later it was found possible to prepare mucosin by hydrolyzing the barium salt for ½ hour on a water bath in an aqueous solution of 1 per cent sulfuric acid. The excess of acid was then removed by means of barium hydroxide and the filtrate concentrated.

Identification of Glucuronic Acid.

The presence of glucuronic acid was demonstrated by the formation of furfural on distillation of the mucoitin sulfuric acid with hydrochloric acid, by the phenylhydrazine derivative of glucuronic acid after the hydrolysis of mucosin with sodium amalgam, and finally by the isolation of the acid potassium salt of saccharic acid on oxidation of mucosin with nitric acid.

The estimation of the yield of phloroglucide of furfural also permitted an approximate estimate of the proportion of glucuronic in the molecule of the mucoitin sulfuric acid.

Distillation with Hydrochloric Acid.—1.5 gm. of barium salt were distilled over flame in 250 cc. of HCl (specific gravity 1.06) until the distillate no longer gave a test with aniline acetate. To the distillate 0.3 gm. of phloroglucide was added and solution allowed to stand over night. The phloroglucine was filtered over a Gooch crucible. Yield was 0.0870 gm., which corresponds to 0.2610 gm. of glucuronic acid. The theory requires 0.5000 gm.

Hydrolysis by Means of Sodium Amalgam.—3.5 gm. of mucosin hydrochloride were dissolved in 50 cc., and 150 gm. of 2 per cent sodium amalgam were added in 25 gm. lots. After each addition, the solution was neutralized with sulfuric acid. After the last portion of amalgam had been added the flask was placed in shaking

machine for 5 hours and then allowed to stand over night. The following day the solution was filtered and neutralized with sulfuric acid. 5 gm. of phenylhydrazine dissolved in 5 cc. of glacial acetic acid were then added and the solution was warmed on a boiling water bath for 30 minutes with reflux. On cooling over night a crystalline deposit formed. This was filtered, suspended in water, again filtered, and suspended in 99.5 per cent alcohol, filtered, and dried in a vacuum desiccator over sulfuric acid.

The melting point of the substance was 125°C. and decomposition with effervescence took place at 132°C. (corrected). A sample prepared from chondrosin had exactly the same melting point. In the communication of Levene and LaForge the melting point was given at 115°. The manner of purification of the substance as carried out at the later date was more rigorous, and the melting point of 125°C. with decomposition at 132° is to be regarded as the correct one.

0.0632 gm. substance gave on combustion 9.4 cc. nitrogen at 28°C. and 767.5 mm. pressure.

	Calculated for $C_{24}H_{26}O_4N_6$ 1.5 H_2O:	Found:
N	17.17	16.92

Oxidation with Nitric Acid.—10 gm. of mucosin hydrochloride were dissolved in 10 cc. of distilled water to which 10 cc. of nitric acid (specific gravity 1.40) were added, and the solution was heated over free flame until the evolution of nitrous acid fumes became very lively. The solution was immediately transferred to a clock glass and evaporated with constant stirring. The subsequent treatment was as usual. The final solution was made up to 10 cc. Of these 1 cc. was used as control, and 9 cc. were allowed to digest with 2 cc. of a 50 per cent solution of potassium hydroxide on a boiling water bath for 2 hours. The solution was then made acid with acetic acid and the acid potassium salt was allowed to crystallize. The crude salt on fractionation out of water yielded a sample of the salt which analyzed as follows:

0.100 gm. salt gave 0.0356 gm. K_2SO_4.

	Calculated for $C_6H_9O_7K$:	Found:
K	15.70	15.95

Estimation and Identification of Acetic Acid.

These experiments aimed to establish the number of acyl groups in the molecule, and to identify the character of the acyl groups.

Quantitative Estimation of Acetic Acid.—2 gm. of the barium salt of mucoitin sulfuric acid were dissolved in 200 cc. of water containing 15 gm. of barium hydroxide and hydrolyzed on a water bath for 5 hours. The product of the reaction was rendered acid to Congo red by means of sulfuric acid and filtered. The solution was then distilled guarding the original volume (600 cc.). The distillate was received in a measured volume of 0.1 N sodium hydroxide. 31.3 cc. of 0.1 N alkali were neutralized by the distillate. Calculated for acetic acid the yield was 0.1878 gm. The theory for one acetyl group requires 0.1944 gm.

Identification of Acetic Acid.—The entire distillate was concentrated under diminished pressure to 8 cc., rendered acid with sulfuric acid, and extracted with ether. To the ethereal extract a few drops of aqueous ammonia were added and the ether was allowed to evaporate spontaneously. The residue was converted into the silver salt.

0.1052 gm. dry substance gave 0.0681 gm. Ag.

	Calculated for $C_2H_3O_2Ag$:	Found:
Ag.....	64.26	64.73

Humor Vitreous Mucoid.

Preparation of Mucoitin Sulfuric Acid.

To humor vitreous of 1,000 eyes enough of a 50 per cent sodium hydroxide solution was added to make the concentration of alkali 3 per cent. The material was allowed to stand 3 days; it was then acidulated and concentrated on a water bath after addition of an excess of barium carbonate. The final product was filtered on suction. To the filtrate enough of basic lead acetate solution was added to precipitate all of the acid. The crude lead salt was washed by decantation, then filtered and the precipitate was washed once with glacial acetic acid. The precipitate was then filtered and washed with alcohol. After this the substance was taken up in water and the mixture rendered slightly alkaline by means of a solution of

potassium hydroxide and the lead salt was decomposed by hydrogen sulfide. From the filtrate hydrogen sulfide was removed by aeration and the solution was poured into 2 liters of alcohol. A precipitate thus formed was washed with alcohol and ether. The substance analyzed as follows:

0.1000 gm. substance required for neutralization 3.54 cc. 0.1 N acid.
0.2000 " gave 0.0528 gm. $BaSO_4$.
0.0958 " Ba salt of the substance gave 0.1208 gm. CO_2 and 0.0490 gm. H_2O.

	Calculated for $C_{26}H_{44}O_{29}N_2S_2Ba_2$:	Found:
C	27.8	34.39
H	3.48	5.72
N	2.32	4.96
S	5.3	3.63
Base	22.7	21.5

Nature of the Amino-Hexose.—6 gm. of the substance were dissolved in 30 cc. of 20 per cent HCl + 1 gm. of stannous chloride + 1 gm. of barium chloride heated with reflux condenser 8 hours over a Babo funnel. The solution was filtered, decomposed with hydrogen sulfide, and freed from barium quantitatively. The final solution was concentrated under diminished pressure to about 5 cc. The sugar began to crystallize in the distilling flask. The entire residue was taken up in methyl alcohol and allowed to crystallize at room temperature. The yield of the substance was 1.0 gm.

0.03018 gm. substance gave 3.47 cc. N at 19°C. and 745 mm. pressure.

	Calculated for $C_6H_{13}O_5N \cdot HCl$:	Found:
N	6.51	6.45

The rotation of the substance was the following:

Initial. Equilibrium.

$$[\alpha]_D^{20} = \frac{+4.60 \times 2.1248}{1 \times 0.1010 \times 1.0018} = +96.77° \qquad \frac{3.43 \times 2.1248}{1 \times 0.1010 \times 1.0018} = +72.15°$$

Furfural Distillation.—1.5 gm. of the barium salt were distilled with 250 cc. of HCl (1.06) so long as distillate showed the presence of furfural. Further treatment was carried out as above, and 0.3 gm. of phloroglucine was added. The yield was 0.1165 gm., which corresponds to 0.3495 gm. of glucuronic acid. The theory required 0.500 gm.

Cornea Mucoid.

Preparation of Mucoitin Sulfuric Acid.

The corneas of 1,000 beef eyes were mechanically separated from the sclera and placed in 1,500 cc. of a 3 per cent sodium hydroxide solution and allowed to stand 3 days, then strained through cheesecloth and acidulated with acetic acid. Barium carbonate was added in excess and all concentrated on a water bath to a thick syrupy mass containing the coagulated protein and the barium carbonate.

The mass was filtered on suction, and to the filtrate sufficient lead carbonate was added to precipitate all the acid. The purification of the lead salt was carried out in the manner described above. The final substance had the following composition.

0.1000 gm. substance required for neutralization 2.42 cc. 0.1 N acid.
0.2000 " " gave 0.0330 gm. $BaSO_4$.

A second precipitate was prepared as follows: The corneas were treated with alkali in the same manner as in the former experiment. The acidulated solution was concentrated in the presence of barium carbonate, and the filtrate poured into an excess of glacial acetic acid. The precipitate was washed repeatedly with glacial acetic acid, then with alcohol. The dry substance was then redissolved in a little water with the aid of potassium hydroxide. The solution was poured into a large excess of 99.5 per cent alcohol. The potassium salt obtained in this manner was dried and analyzed.

0.200 gm. substance required for neutralization 6.90 cc. 0.1 N acid.
0.200 " " gave on fusion 0.0452 gm. $BaSO_4$.
0.0950 " potassium salt substance gave 0.1318 gm. CO_2 and 0.0528 gm. H_2O.

	Calculated for: $C_{26}H_{44}O_{29}N_2S_2Ba_2$:	Found: I	Found: II
C	27.8		37.83
H	3.48		6.32
N	2.32	3.93	4.62
S	5.30	2.27	3.10
Base	22.70		6.55

Hydrolysis of the Substance for Amino-Hexose.—4.5 gm. of the substance were hydrolyzed in 60 cc. of 20 per cent hydrochloric acid, together with 1 gm. of barium chloride and 1 gm. of stan-

nous chloride, and the solution was heated with reflux condenser for 12 hours over a Babo funnel. The solution as usual turned dark brown. It was diluted with an equal volume of water and was then freed from tin by means of hydrogen sulfide, and from barium by means of sulfuric acid. The solution was concentrated to a thick syrup. Glucosamine crystallized in the distilling flask. It was taken up in methyl alcohol and kept at room temperature in order to complete crystallization. The yield was 0.520 gm. The appearance of the crystals under the microscope was typical for glucosamine. The substance turned brown at about 200°C. and turned black at 220°C. It did not melt. 0.020 gm. of the substance gave in the Van Slyke apparatus 2.41 cc. of nitrogen gas at 27°C. and 756.8 mm. pressure.

	Calculated for $C_6H_{13}O_5N.HCl$:	Found:
N.....	6.51	6.80

The substance had the following rotation:

Initial.

$$[\alpha]_D^{20} = \frac{+ 2.27 \times 2.0390}{1 \times 0.0485} = + 95.43°$$

Equilibrium.

$$\frac{+ 1.71 \times 2.0390}{1 \times 0.485} = + 71.89°$$

Group II B.

Mucin of the Gastric Mucosa.

Preparation of Mucoitin Sulfuric Acid.

Mucus was removed from the gastric wall mechanically and a concentrated solution of barium hydroxide was added to make the total solution contain 3 per cent of the hydroxide. The solution was allowed to stand 3 days at room temperature. At the end of this time the solution was rendered acid to Congo red by means of sulfuric acid, then centrifugalized. The supernatant liquid was neutralized with a solution of barium hydroxide until neutral to Congo red, but still acid to litmus, and finally neutralized to litmus by means of barium carbonate, then boiled for about 3 hours, and filtered. To the filtrate again barium carbonate was added and the mixture was allowed to stand on a water bath from 2 to 3 days until

a sample of the filtrate showed a negative biuret test. This was centrifugalized and the supernatant liquid precipitated by means of glacial acetic acid. The precipitate was redissolved in water and reprecipitated by means of glacial acetic acid. The precipitate thus formed was repeatedly washed with 95 per cent alcohol until most of the glacial acetic acid was removed. This material was then dissolved in a minimum amount of water, the solution was exactly neutralized with a solution of barium hydroxide, and the barium salt of mucoitin sulfuric acid precipitated by means of alcohol. The crude salt was repeatedly washed with a 50 per cent solution of alcohol until most of the inorganic impurities were removed, then with alcohol of progressively increasing concentration. This salt was a mixture of mucoitin sulfuric and nucleic acid. To separate the two the mixture was taken up in water and centrifugalized. The salt of the nucleic acid, being insoluble, was removed in this manner. To complete separation it was necessary to repeat the operation several times. Finally the clear solution was poured into an excess of alcohol giving a precipitate of the barium salt of the mucoitin sulfuric acid. A sample of the material prepared in this manner had the following composition.

0.1000 gm. substance required for neutralization 2.48 cc. 0.1 N acid.
0.1500 " " gave 0.0162 gm. $BaSO_4$.

	Calculated for $C_{26}H_{44}O_{29}N_2S_2Ba_2$:	Found:
N	2.32	3.47
S	5.30	1.48

$$[\alpha]_D^{20} = \frac{-0.19 \times 5.3035}{1 \times 0.0447} = -22.54°$$

Preparation of Mucosin.—For the preparation of mucosin 14.0 gm. of the barium salt were dissolved in 100 cc. of water and 15 cc. of concentrated hydrochloric acid, and allowed to stand on the boiling water bath 20 minutes. The solution was concentrated under diminished pressure (the temperature of the bath not exceeding 40°C.) to a volume of 5 cc. The solution was poured into a solution of 1 liter each of 99.5 per cent alcohol and ether. A precipitate formed which was removed by filtration. The precipitate

was then dissolved in about 3 cc. of water and precipitated by 400 cc. of 99.5 per cent alcohol. To filtrate, an equal volume of ether was added and thus a second precipitate was formed which reduced Fehling's solution, but was not yet free from mucoitin.

0.010 gm. in the Van Slyke apparatus gave 0.38 cc. nitrogen at 22° and 753.7 mm. pressure.

	Calculated for $C_{12}H_{21}O_{11}N.HCl$:	Found:
NH_2-N	3.58	2.12

The optical rotation of the substance was as follows:

$$[\alpha]_D^{20} = \frac{+ 0.23 \times 5.8780}{1 \times 0.0525} = + 25.75°$$

Glucuronic Acid.—The presence of glucuronic acid was demonstrated by furfural distillation. 1.0065 gm. were distilled with 250 cc. of hydrochloric acid (specific gravity 1.06). The yield of phloroglucine was 0.0440 gm. The theory requires 0.100 gm.

Hydrolysis by Sodium Amalgam.—7 gm. of mucosin prepared as above were dissolved in 100 cc. of water and 200 gm. of a 2 per cent amalgam were added in portions of 25 gm. at intervals. The entire operation lasted 24 hours. Before each new addition of amalgam the solution was neutralized with sulfuric acid. The final product was separated from mercury and filtered. To this solution were added 7 cc. of phenylhydrazine dissolved in 7 cc. of glacial acetic acid, and the entire solution was allowed to stand for 30 minutes with reflux on a boiling water bath. The reaction product was filtered from tar and allowed to stand at 0°C. over night. A small crystalline deposit formed. There was not sufficient material for purification or analysis.

Acetyl Estimation.—2 gm. of the barium salt were, with 300 cc. of water and 15 gm. of barium hydroxide, allowed to hydrolyze 5 hours, then neutralized, and the acetic acid was distilled into a 0.1 N solution of sodium hydroxide. The acid neutralized 26 cc. of the 0.1 N alkali, which corresponds to 0.156 gm. of acetic acid. The theory for one acetyl group requires 0.194 gm. The distillate was concentrated to very small volume. This was acidulated with sul-

furic acid and extracted with ether. From this the silver salt was obtained. It analyzed as follows:

0.1032 gm. substance gave 0.0662 gm. Ag.

	Calculated for $C_2H_4O_2Ag$:	Found:
Ag	64.14	64.26

Serum Mucoid.

Preparation of Mucoitin Sulfuric Acid.

The mucoitin sulfuric acid from this mucoid was prepared on one occasion by treatment of the entire serum, and on the other, by treatment of the protein obtained from the serum on coagulation by boiling.

The first sample was prepared in the following way. To 12.5 liters of the serum a 50 per cent solution of NaOH was added until the solution contained 3 per cent of alkali. It was allowed to stand 3 days at 40°C., then rendered acid by means of acetic acid, and concentrated on a water bath in presence of excess of $BaCO_3$. The filtrate was converted into the lead salt. This was treated with glacial acetic acid, dried with alcohol, freed from lead, and again reprecipitated with lead acetate. The lead salt was repeatedly washed in a mortar with glacial acetic acid. Finally it was washed with alcohol and dried. The yield of the dry substance was 14.0 gm. The substance contained 5.10 per cent nitrogen. This sample was used for hydrolysis.

The second sample was prepared from the coagulum obtained from 12.5 liters of beef serum. The process of preparation was exactly as in the above experiment. The lead salt was converted into the barium salt. The lead salt was suspended in water, and excess of barium carbonate was added, and hydrogen sulfide gas passed until all lead separated out. From the filtrate the hydrogen sulfide was removed by aeration. The solution was finally precipitated by means of 99.5 per cent alcohol. The precipitate was then dissolved in a little water and the mixture was centrifugalized to remove all insoluble Ba salts. This operation was repeated several times. Finally the solution was precipitated by means of 99.5 per

cent alcohol. This substance was dried and yielded 1.5 gm. Analysis gave the following values.

0.1000 gm. substance required for neutralization 3.75 cc. 0.1 N acid.
0.1961 " " gave on fusion 0.0279 gm. $BaSO_4$.
0.1008 " " " " combustion 0.1098 gm. CO_2 and 0.0400 gm. H_2O.
0.1961 " " treated with sulfuric acid gave 0.0971 gm. $BaSO_4$.

	Calculated for $C_{26}H_{44}O_{29}N_2S_2Ba_2$:	Found:
C	27.80	29.71
H	3.48	4.44
N	2.32	5.25
S	5.30	1.96
Ba	22.74	29.14

13 gm. of the first sample were taken up in 80 cc. of a 20 per cent solution of hydrochloric acid. 2 gm. of barium chloride and 2 gm. of stannous chloride were added. The solution was heated with reflux condenser 13 hours. The dark brown solution was filtered from melanin and diluted with an equal volume of water. The solution was freed from lead and tin by means of hydrogen sulfide and from barium by sulfuric acid.

The solution was concentrated under diminished pressure nearly to dryness. The residue was taken up in methyl alcohol and allowed to stand. Typical crystals of glucosamine hydrochloride separated out.

0.020 gm. in the Van Slyke apparatus gave 2.23 cc. nitrogen at 25°C. and 759.6 mm. pressure.

	Calculated for $C_6H_{13}O_5N.HCl$:	Found:
N	6.51	6.19

Initial.

$$[\alpha]_D^{25} = \frac{+ 1.78 \times 2.0355}{1 \times 0.0405} = + 89.46°$$

Equilibrium.

$$[\alpha]_D^{25} = \frac{+ 1.42 \times 2.0355}{1 \times 0.0405} = + 71.37°$$

The substance began to turn brown at 200°C. and turned black at 220°C. It did not melt.

[Reprinted from The Journal of the American Chemical Society, October, 1918, Vol. xl, No. 10, pp. 1580–1590.]

ON NITRO- AND AMINOARYL ARSONIC ACIDS.

By WALTER A. JACOBS, MICHAEL HEIDELBERGER, and IDA P. ROLF.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received July 9, 1918.)

In the course of studies on the synthesis of organic arsenic compounds for therapeutic purposes it was found necessary to prepare a number of aminoaryl arsonic acids. It was therefore of importance to find methods which would furnish these substances in amounts sufficient for synthetic work. Of the methods available the application of the Béchamp synthesis, first used for the preparation of arsanilic acid, has rendered directly accessible only a limited number, mainly *p*-aminoaryl arsonic acids. In order to obtain *o*- and *m*-amino arsonic acids it has usually been necessary to employ indirect methods.

The most useful of these involves the preparation of the corresponding nitro compound, which is then reduced to the desired amino acid. In case the nitro acid is synthesized by direct nitration of an aryl arsonic acid, only the *m*-nitro compound can be obtained. More general, however, is Bart's excellent method[1] in which a diazo or isodiazo group is replaced by the arsonic acid residue. This reaction has greatly increased the availability of aromatic arsonic acids, many of which have been unobtainable by other means. Among the wide variety of examples given by Bart are enough to demonstrate the value of the method for the preparation of the nitro arsonic acids and his procedure was, in fact, used later in a few instances by others[2] for the same purpose.

In the present work we have found the method to be of exceptional service, particularly when *o*- and *p*-nitro amines were used as starting material. The reaction gave excellent yields and permitted the preparation of fairly large amounts of the *o*- and *p*-nitroaryl arsonic acids.

[1] Bart, D. R. P. 250, 264.

[2] D. R. P. 266, 944; 267, 307; Bauer, *Ber.*, 48, 1582 (1915).

In the case of the *m*-nitro amines, however, the reaction between the alkaline diazo solution and sodium arsenite was far from being a smooth one, side reactions apparently disturbing its course. Under the conditions employed *m*-nitraniline, for instance, was found to yield but a trace of *m*-nitrophenylarsonic acid. The results were better in the case of the *m*-nitrotolylamines, particularly *p*-nitro-*o*-toluidine, but in no case was the yield as good as those obtained in the *o*- and *p*-series.

Once in possession of the nitro acid the next step is the reduction of the nitro to the amino group without affecting the arsonic acid residue. The principal reagents which have been employed for the selective reduction of the nitro group in these compounds are ammonium sulfide, sodium hydrosulfite, sodium amalgam, and ferrous hydroxide. Ammonium sulfide has the disadvantage of attacking the arsenic residue, requiring subsequent removal of combined sulfur. Sodium hydrosulfite, although frequently used, requires careful control and it has often been found difficult to avoid simultaneous reduction of the arsenic acid group. Sodium amalgam has also given good results, but it is scarcely the most convenient reagent for preparative purposes if a better substitute is available.

On the other hand, ferrous hydroxide in an alkaline medium, first employed by Benda[1] for the preparation of *p*-phenylenediamine arsonic acid and later for other reductions,[2] has proven a very reliable selective reducing agent. We believe that this fact should be emphasized more than has been done in the past, since we have found it to give most excellent results in the reduction of all the nitro arsonic acids which we have prepared. In our study of the method, the remarkable specificity of this reagent for the nitro group was demonstrated by the fact that even if an excess of ferrous salt was employed the yield of amino compound in practically every case was 80% of the theory or better. In view of these facts the method seems directly indicated for the reduction of all nitroaryl arsonic acids which do not contain groups incompatible with strong alkali.

[1] *Ber.*, **44**, 3302 (1911).
[2] *Ibid.*, **47**, 1006, 1316 (1914).

EXPERIMENTAL PART.

Since the reduction of the nitro arsonic acids with ferrous sulfate was carried out under identical conditions in each case, the procedure used by us is given here to avoid its frequent repetition.

Seven molecular equivalents (based on the amount of nitro arsonic acid) of ordinary ferrous sulfate are dissolved in about 3 parts of warm water, chilled well, and transferred to a wide-mouthed bottle fitted with a rubber stopper; 25% sodium hydroxide solution is then added until the ferrous hydroxide mud reacts strongly alkaline to litmus paper on vigorous shaking. The use of a weak base, such as ammonia,[1] results in poor yields of the amino acid since ammonia is too weakly alkaline to decompose the insoluble iron salt formed by the arsonic acid during the reduction. A solution of the nitro acid in dil. sodium hydroxide is then poured in at once and the mixture vigorously shaken for 5 minutes. The pale blue hydroxide immediately changes in color to the dark brown of ferric hydroxide. Warming the mixture is unnecessary and diminishes the yield. The mud is poured on to a large Büchner funnel, the residue being finally washed with water. As this filtration is slow it is necessary to use a large funnel to avoid a troublesome delay. The isolation of the amino acid from the filtrate depended on the properties of the compound and is described separately for each case.

Arsenic was determined in each substance by decomposing 0.25 to 0.3 g. by the Kjeldahl method, using 25 cc. of sulfuric acid, 10 g. of potassium sulfate, and a very small crystal of copper sulfate. After destruction of the organic matter and dilution with water the arsenic was precipitated by hydrogen sulfide and determined gravimetrically in the usual way as magnesium pyroarsenate.

o-Nitrophenylarsonic Acid, $o\text{-}O_2NC_6H_4AsO_3H_2$.—The preparation of this substance has been described by Bart,[2] but we believe the method used below to be more convenient. Moreover, the description of the substance in the patent is incomplete and inaccurate.

One hundred grams of *o*-nitraniline (prepared by the excellent method of Witt and Utermann[3]) was ground under 500 cc. of 1 : 1

[1] This Journal, 39, 1435 (1917).

[2] *Loc. cit.*

[3] *Ber.*, 39, 3901 (1906).

hydrochloric acid, the mixture chilled to 10°, and diazotized with a solution of 55 g. of sodium nitrite. After 10 to 15 minutes' stirring the solution was filtered from traces of undissolved nitraniline and poured slowly, with shaking, into 550 cc. of 25% sodium hydroxide solution, keeping the temperature below 0°. The alkaline solution was then added to 135 g. of sodium arsenite dissolved in 1250 cc. of water and the mixture heated to 60–70° for 1.5 to 2 hours, during which a slow but steady nitrogen evolution occurred. Overheating is particularly to be avoided. After acidification with a slight excess of acetic acid the dark-colored solution was boneblacked and hydrochloric acid added to the deep yellow filtrate until strongly acid to congo red. On thorough chilling the nitrophenylarsonic acid crystallized as a heavy, pale yellow powder. The yield was 110 grams.

Recrystallized from water the nitro acid separates as pale yellow, glistening, hexagonal plates which contain one molecule of water of crystallization. The anhydrous acid melts and decomposes at 235–40° with preliminary softening, and does not merely intumesce as stated by Bart. The anhydrous acid is difficultly soluble in cold alcohol but dissolves on warming and is very sparingly soluble in chloroform or acetone. The hydrate is sparingly soluble in cold water but dissolves easily on warming. It is partly soluble in acetic acid but again crystallizes out and is then practically insoluble, presumably due to dehydration.

Subst., air dry, 0.6741 g. *in vacuo* at 100° over H_2SO_4; loss, 0.0469 g.
Calc. for $C_6H_6O_5NAs.H_2O$: H_2O, 6.80. Found: 6.96.
Anhydrous: 0.1384 g. subst.; 7.0 cc. N (24.5°, 757 mm.); 0.3197 g. subst.; 0.1972 g. $Mg_2As_2O_7$.
Calc. for $C_6H_6O_5NAs$: N, 5.67; As, 30.32%. Found: N, 5.78; As, 29.77.

o-Aminophenylarsonic Acid (*o-Arsanilic Acid*), $o\text{-}H_2NC_6H_4AsO_3H_2$.— Forty grams of *o*-nitrophenylarsonic acid was reduced with 300 g. of ferrous sulfate by the standard method given above. The alkaline filtrate was acidified with hydrochloric acid, warmed, and treated with concentrated barium chloride solution until the sulfuric acid was completely removed, avoiding at the same time an undue excess of barium chloride. The filtrate was rendered slightly alkaline and concentrated to small bulk *in vacuo*. The precipitate of sodium chloride

was filtered off and washed with a small amount of saturated sodium chloride solution. On treating the filtrate, the volume of which was about 200 cc., with hydrochloric acid until congo red paper just began to turn, the *o*-arsanilic acid rapidly crystallized, forming a thick mass of needles. After thorough chilling the acid was filtered off and washed with ice water. The yield was 27 g., or 80% of the theory. By using ferrous chloride instead of ferrous sulfate the treatment with barium chloride would of course become unnecessary.

The substance is quite soluble in water and melts at 153° with slight preliminary softening. The diazotized solution couples with R-salt to form a not very intense reddish orange dye, the *m*- and *p*-isomers giving deeper shades. In other properties it corresponded to those given by Benda,[1] who obtained the acid by a more involved method.

Subst., 0.1456 g.; 8.3 cc. N (23.0°, 758 mm.).
Calc. for $C_6H_8O_3NAs$: N, 6.45. Found: 6.56.

m-Aminophenylarsonic Acid (*m-Arsanilic Acid*).—Sixty-three grams of *m*-nitrophenylarsonic acid (prepared without difficulty by nitrating phenylarsonic acid according to Michaelis[2]) was reduced with 500 g. of ferrous sulfate as previously described. Owing to the sparing solubility of *m*-arsanilic acid in water it sufficed to acidify the filtrate strongly with acetic acid, whereupon the amino acid separated rapidly as a yellow, crystalline powder. The yield was 47.5 grams. It was recrystallized by dissolving in dilute ammonia, boneblacking, and reprecipitating with acetic acid, separating as practically colorless, stout, rhombic prisms which melt at 213–5° with gas evolution. We have nothing to add to the description given by Bertheim.[3]

Subst., 0.1304 g.; 7.4 cc. N (22.5°, 766 mm.).
Calc. for $C_6H_8O_3NAs$: N, 6.45. Found: 6.61.

p-Nitrophenylarsonic Acid.—This substance is mentioned by Bart in D. R. P. 250,264, but no description is given. The following procedure was used by us for its preparation:

27.6 g. of *p*-nitraniline was dissolved in 400 cc. of hot, 10% hydrochloric acid, chilled to 10°, and diazotized with a solution of 14.4 g. of

[1] *Ber.*, **44**, 3304 (1911).
[2] *Ann.*, **320**, 294 (1902).
[3] Bertheim, *Ber.*, **41**, 1657 (1908); Bertheim and Benda, *Ber.*, **44**, 3297 (1911).

sodium nitrite. After 15 minutes the solution was poured into 460 cc. of 10% sodium hydroxide, keeping the temperature at 0°. After the addition of 40 g. of sodium arsenite dissolved in water the mixture was heated to 55–65° until evolution of nitrogen ceased. After acidification with acetic acid and boneblacking, the filtrate was concentrated to small bulk *in vacuo* and acidified to congo red with hydrochloric acid. The nitro acid separated quickly as a light yellow precipitate and was washed with ice water. The yield was 18 grams. Recrystallized from water, the nitro acid separates as pale yellow aggregates of minute leaflets which do not melt below 275°. It is sparingly soluble in hot methyl alcohol or acetic acid and also difficultly soluble in the cold in water, alcohol, or 50% alcohol, but dissolves readily in these solvents on heating.

Subst., 0.1422 g.; 7.4 cc. N (25.0°, 757 mm.).
Calc. for $C_6H_6O_5NAs$: N, 5.67. Found: 5.94.

p-Aminophenylarsonic Acid (*p-Arsanilic Acid*).—This important substance is most easily obtained by the direct arsenation of aniline, but as the reduction with ferrous hydroxide was carried out as a test of the method it is given here. Fifteen grams of *p*-nitrophenylarsonic acid was reduced with 125 g. of ferrous sulfate. Since arsanilic acid is only incompletely precipitated from large volumes the sulfate ion was replaced by the chloride ion with barium chloride as in the case of *o*-arsanilic acid. The filtrate from the barium sulfate was made slightly alkaline and concentrated to small bulk *in vacuo*. On careful treatment with hydrochloric acid the filtrate from the sodium chloride deposited arsanilic acid in 80% of the theory. Recrystallized from water it separated in characteristic form.

Subst., 0.2215 g.; Kjeldahl, 9.85 cc. 0.1 *N* HCl.
Calc. for $C_6H_8O_3NAs$: N, 6.45. Found: 6.23.

2-Nitro-4-methylphenylarsonic Acid (*4-Methyl-6-nitrophenylarsonic Acid*).—Twenty-two grams of 3-nitro-4-toluidine, 3, 4-$(O_2N)(H_2N)C_6H_3CH_3$, was pulverized under 100 cc. of 20% hydrochloric acid and diazotized at 10° with 11 g. of sodium nitrite. When solution was practically complete the diazo mixture was poured into 355 g. of 10% aqueous sodium hydroxide kept at 0°. A concentrated solution of 27 g.

of sodium arsenite was added at once and the mixture heated at 60–70° for about 1.5 to 2 hours. After acidifying with acetic acid and boneblacking, the filtrate was acidified to congo red with hydrochloric acid and deposited the arsonic acid at once as a light yellow, crystalline powder. The yield was 22 grams. On dissolving in hot sodium acetate solution and acidifying again to congo red the nitro acid separates rapidly as faintly yellow, minute rods melting and decomposing at about 255–60°. It is very sparingly soluble in cold water, alcohol, or acetic acid, but is appreciably soluble in these solvents on boiling.

Subst., 0.1172 g.; 5.6 cc. N (26.0°, 763 mm.); subst., 0.3116 g.; 0.1858 g. $Mg_2As_2O_7$.
Calc. for $C_7H_8O_5NAs$: N, 5.36; As, 28.70. Found: N, 5.48; As, 28.79.

2-Amino-4-methylphenylarsonic Acid (*4-Methyl-6-aminophenylarsonic Acid*).—14.5 g. of 2-nitro-4-methylphenylarsonic acid was reduced with 120 g. of ferrous sulfate. The acidified filtrate was treated with barium chloride as in the case of *o*-arsanilic acid and the filtrate made slightly alkaline and concentrated to small bulk *in vacuo*. The insoluble sodium chloride was filtered off and the amino acid precipitated by the careful addition of hydrochloric acid. The yield was 10 grams. Recrystallized from a small volume of hot water the amino acid forms colorless needles which melt at 180° with preliminary sintering and darkening. It is easily soluble in methyl or ethyl alcohol or glacial acetic acid and but sparingly in acetone or ether, and is appreciably soluble in water at room temperature. Its solution in dil. hydrochloric acid diazotizes smoothly and couples with R-salt to form a weak, orange-red dye as in the case of *o*-arsanilic acid.

Subst., 0.2382 g.; Kjeldahl, 10.25 cc. 0.1 *N* HCl; subst., 0.2803 g.; 0.1870 g. $Mg_2As_2O_7$.
Calc. for $C_7H_{10}O_3NAs$: N, 6.06; As, 32.44. Found: N, 6.03; As, 32.20.

2-Nitro-6-methylphenylarsonic Acid (*2-Methyl-6-nitrophenylarsonic Acid*).—Twenty-two grams of 3-nitro-*o*-toluidine, 2,3-$(H_2N)(O_2N)C_6H_3CH_3$, was diazotized and treated with sodium arsenite exactly as in the case of 3-nitro-4-toluidine. The yield of the resulting nitroarsonic acid was 19 grams. Recrystallized from hot water it sepa-

rates slowly as star-shaped aggregates of pale yellow, delicate needles which decompose at 228–30° with preliminary softening and darkening. It is sparingly soluble in hot water and practically insoluble in the cold, but is somewhat more soluble in boiling acetic acid and readily in hot alcohol. It dissolves in methyl alcohol at room temperature.

Subst., 0.1603 g.; 7.6 cc. N (25.0°, 761 mm.); subst., 0.3110 g.; 0.1837 g. $Mg_2As_2O_7$. Calc. for $C_7H_8O_5NAs$: N, 5.36; As, 28.70. Found: N, 5.44; As, 28.50.

2-Amino-6-methylphenylarsonic Acid (*2-Methyl-6-aminophenylarsonic Acid*).—8.5 g. of 2-nitro-6-methylphenylarsonic acid was reduced with 70 g. of ferrous sulfate and the resulting amino acid isolated exactly as in the case of *o*-arsanilic acid. The yield was 5 grams. Recrystallized from a small volume of hot water the aminotolylarsonic acid separates slowly as rosets or plates which decompose at 175–80°. It is soluble in methyl or ethyl alcohol or glacial acetic acid, less so in acetone or water at room temperature. A solution in hydrochloric acid is readily diazotized but the diazo solution does not give a strong color with R-salt.

Subst., 0.1658 g.; Kjeldahl, 7.3 cc. 0.1 *N* HCl.
Calc. for $C_7H_{10}O_3NAs$: N, 6.06. Found: 6.16.

3-Nitro-4-methylphenylarsonic Acid (*4-Methyl-5-nitrophenylarsonic Acid*).—This substance was prepared by Michaelis[1] by the nitration of *p*-tolylarsonic acid. As in the case of other *m*-nitro amines, the application of the Bart reaction to 2-nitro-4-toluidine, 2,4-$(O_2N)(H_2N)C_6H_3CH_3$, did not give as good results as in the *o*- and *p*-series. Many modifications of the method were tried but the best that could be obtained was unsatisfactory from the standpoint of yield.

Sixty grams of 2-nitro-4-toluidine was dissolved in 400 cc. of hot 1 : 1 hydrochloric acid, poured into one liter of cold water, chilled to 10°, and the mixture then treated with a concentrated solution of 30 g. of sodium nitrite. After complete diazotization the solution was poured into 600 cc. of 25% aqueous sodium hydroxide, keeping the temperature at 0°. A solution of 100 g. of sodium arsenite was then added and the mixture allowed to stand overnight at room tempera-

[1] *Ann.*, 320, 321 (1902).

ture. A steady evolution of nitrogen occurred, but a large amount of insoluble tar was formed which consisted largely of nitrotoluene. After acidifying the mixture with acetic acid and boneblacking, the filtrate was concentrated to small bulk *in vacuo* and acidified to congo red with hydrochloric acid. The nitro acid separated rapidly as lustrous crystals which were purified by dissolving in dilute sodium acetate solution and acidifying the filtrate with hydrochloric acid. The yield was only 16 grams. Recrystallized from a small volume of hot water the nitro acid separates as cream-colored needles which do not melt below 285°. It is sparingly soluble in cold water or acetic acid, easily on boiling. It dissolves readily in methyl or ethyl alcohol and very sparingly in acetone or chloroform.

Subst., 0.1422 g.; 6.8 cc. N (25.0°, 764 mm.); subst., 0.3361 g.; 0.2015 g. $Mg_2As_2O_7$. Calc. for $C_7H_8O_5NAs$: N, 5.36; As, 28.70. Found: N, 5.51; As, 28.93.

3-Amino-4-methylphenylarsonic Acid (*4-Methyl-5-aminophenylarsonic Acid*).—Ten grams of 3-nitro-4-methylphenylarsonic acid was reduced as usual with 80 g. of ferrous sulfate. Owing to the solubility of the amino acid the method used for the isolation of *o*-arsanilic acid was found necessary. The concentrated filtrate from the barium sulfate yielded 7 g. of the aminotolyl arsonic acid on careful acidification with hydrochloric acid. Recrystallized from a small volume of hot water it separates very slowly as woolly masses of delicate, microscopic needles which soften and melt at 172–5° to a liquid filled with bubbles. It is somewhat soluble in water, methyl or ethyl alcohol, or acetic acid at room temperature, and dissolves readily in these on warming. The hydrochloric acid solution is smoothly diazotizable, giving a cherry-red color with R-salt.

Subst., 0.2127 g.; Kjeldahl, 9.3 cc. 0.1 *N* HCl; subst., 0.3057 g.; 0.2027 g. $Mg_2As_2O_7$. Calc. for $C_7H_{10}O_3NAs$: N, 6.06; As, 32.44. Found: N, 6.12; As, 32.00.

3-Nitro-6-methylphenylarsonic Acid (*2-Methyl-5-nitrophenylarsonic Acid*).—This substance has recently been described by Karrer,[1] who obtained it by the direct nitration of *o*-tolylarsonic acid. By the Bart reaction 4-nitro-*o*-toluidine ($2,4\text{-}(H_2N)(O_2N)C_6H_3CH_3$) was found to yield the same product more conveniently. Being a *m*-

[1] *Ber.*, 48, 311 (1915).

nitro compound 4-nitro-*o*-toluidine did not react as smoothly as the *o*- and *p*-nitro amines, but the reaction proceeded more normally than in the case of 2-nitro-4-toluidine, described above.

Sixty-six grams of 4-nitro-*o*-toluidine was dissolved in 440 cc. of hot 1 : 1 hydrochloric acid and poured into 1100 cc. of water. The mixture was diazotized at 10° with a solution of 33 g. of sodium nitrite and the diazo solution then poured into 660 cc. of 25% sodium hydroxide solution, keeping the temperature below 0°. After adding a concentrated solution of 110 g. of sodium arsenite and letting stand overnight at room temperature the mixture was acidified with acetic acid and the tar collected with boneblack. The filtrate was concentrated to small bulk *in vacuo* and acidified to congo red with hydrochloric acid. Forty grams of the nitro acid separated as light yellow, glistening plates. As so obtained the substance was contaminated with some amorphous material which depressed the melting point but did not interfere with the reduction to the amino acid. For analysis the nitro acid was dissolved in dil. sodium acetate solution and reprecipitated with hydrochloric acid after boneblacking in the cold. When rapidly heated it often melts completely at about 225°, evolves gas, and resolidifies, finally melting with decomposition at 261–3°. The same behavior was observed on plunging the substance into a bath heated to 230°, but if the acid is heated slowly it sinters and changes its appearance at about 220°, but does not melt until 261–3° is reached. As Karrer described the acid as darkening at 230° and melting at 261° some of the substance was synthesized by his method and was found to behave in the same way as our preparation. It is probable that at the melting point of the nitro acid, *i. e.*, about 225°, water is liberated with the formation of the anhydride, which then melts at 261–3°. The acid is very sparingly soluble in cold water, alcohol, or acetic acid, but dissolves in these on heating. It is soluble in methyl alcohol and practically insoluble in acetone or chloroform. Whereas the substance prepared by Karrer's method is colorless a yellow color persists in the compound as obtained by the Bart method.

Subst., 0.1468 g.; 6.9 cc. N (23.0°, 756 mm.); subst., 0.2244 g.; 0.1346 g. $Mg_2As_2O_7$. Calc. for $C_7H_8O_5NAs$ N, 5.36; As, 28.70. Found: N, 5.39; As, 28.93.

3-Amino 6-methylphenylarsonic Acid (*2-Methyl-5-aminophenylarsonic Acid*).—Thirty-seven grams of the above nitrotolylarsonic acid was reduced with 300 g. of ferrous sulfate. On acidification of the filtrate with acetic acid the amino acid separated on scratching. The crude product was purified by dissolving in dilute hydrochloric acid and reprecipitating with sodium acetate solution, the yield being 80% of the theory. Recrystallized from hot water it separates as cream-colored prisms which decompose at about 235–45° with preliminary darkening. It is rather difficultly soluble in boiling water and still less so in boiling alcohol, methyl alcohol, or acetic acid. It is smoothly diazotized in acid solution, giving a deep cherry-red color with R-salt.

Subst., 0.1425 g.; 7.6 cc. N (24.0°, 755 mm.).
Calc. for $C_7H_{10}O_3NAs$: N, 6.06. Found: 6.09.

4-Nitro-6-methylphenylarsonic Acid (*2-Methyl-4-nitrophenylarsonic Acid*).—Fifteen grams of 5-nitro-*o*-toluidine, 2, 5-$(H_2N)(O_2N)C_6H_3CH_3$, was boiled with 80 cc. of 1 : 1 hydrochloric acid and then poured, with turbining, into 200 cc. of cold water. The solution was diazotized at 10° with 7.2 g. of sodium nitrite and the mixture poured into 100 g. of 25% aqueous sodium hydroxide, keeping the temperature below 0°. A solution of 20 g. of sodium arsenite was then added and the mixture warmed gently until the evolution of nitrogen ceased. It is important not to allow the temperature to exceed 70°, as the resulting nitrotolylarsonic acid will then be contaminated with by-products. After acidification with acetic acid and boneblacking, the filtrate was treated with hydrochloric acid, the nitro compound quickly separating as minute, almost colorless needles which melt and decompose at about 235–40°. The yield was 14 grams. The compound is sparingly soluble in cold water, alcohol, or acetic acid, but dissolves in these on boiling. It is practically insoluble in acetone or chloroform.

Subst., 0.1341 g.; 6.4 cc. N (25.0°, 757 mm.); subst., 0.3049 g.; 0.1795 g. $Mg_2As_2O_7$.
Calc. for $C_7H_8O_5NAs$: N, 5.36; As, 28.70. Found: N, 5.45; As, 28.41.

4-Amino-6-methylphenylarsonic Acid (*2-Methyl-4-aminophenylarsonic Acid, m-Toluidine Arsonic Acid*).—Fifteen grams of the nitro

acid was reduced with 120 g. of ferrous sulfate. On treating the filtrate with hydrochloric acid until congo red just began to turn, 11 g. of the amino acid quickly separated. As the volume of the liquid is quite large it is essential to add neither too much nor too little hydrochloric acid. This difficulty is obviated, however, by isolating the acid by the method used in the case of *o*-arsanilic acid. Recrystallized from hot water, the acid separates as flat, microscopic needles and pointed prisms which darken and decompose at 222–4°. Since this melting point did not correspond with that given by Benda and Kahn[1] (180°) for the substance as obtained by the direct arsenation of *m*-toluidine, we prepared the compound by this method. The amino acid as so obtained also melted at 222–4°, as did a mixture of the two. The substance is easily diazotized, coupling with R-salt to give an orange-red solution.

Subst., 0.1345 g.; 7.0 cc. N (22.0°, 768 mm.).
Calc. for $C_7H_{10}O_3NAs$: N, 6.06. Found: 6.09.

4-Nitro-2,5-dimethylphenylarsonic Acid (*2,5-Dimethyl-4-nitrophenylarsonic Acid*).—8.2 g. of *p*-nitro-*p*-xylidine (2,5-dimethyl-4-nitraniline) was ground up in a mortar with 25 cc. of concd. hydrochloric acid. The mixture was washed into a beaker with about 40 cc. of water and diazotized at 10° with 3.5 g. of sodium nitrite. The mixture was allowed to reach 15°, stirring continuously, and although a precipitate of apparently unchanged material remained, the whole was nevertheless poured into 180 cc. of 10% sodium hydroxide solution, keeping the temperature below 0°. 9 g. of sodium arsenite in a little water were then added, after which the mixture was heated to 50°. When the resulting gentle evolution of nitrogen had almost ceased the temperature was raised to 60–70° for one and one-half hours longer. After acidifying with acetic acid and boneblacking, the filtrate was acidified to congo red with hydrochloric acid, causing immediate separation of the nitro arsonic acid. This was allowed to stand in the ice box, filtered off, washed with water, and dried. The yield was 4.8 g. A portion was recrystallized from 50% acetic acid, separating as pale yellow, spindle-shaped prisms, wedge-shaped platelets, and long, flat plates which melt and decompose at about

[1] *Ber.*, **41**, 1675 (1908).

290°, with preliminary softening and darkening. It is therefore not identical with the nitro-*p*-xylenearsonic acid obtained by Michaelis[1] by nitrating *p*-xylenearsonic acid. This substance forms needles melting at 205° and from its mode of formation would seem to be the 3-nitro isomer of the acid here described. The 4-nitro acid is sparingly soluble in boiling water or cold 50%, or glacial, acetic acid, but dissolves readily in the last two on heating. It is also soluble in boiling alcohol.

Subst., 0.1409 g.; 6.4 cc. N_2 at 30.0° and 757 mm.
Subst., 0.2943 g.; 0.1648 g. $Mg_2As_2O_7$.
Calc. for $C_8H_{10}O_5NAs$: N, 5.09; As, 27.24. Found: N, 5.10; As, 27.03.

4-Amino-2,5-dimethylphenylarsonic Acid (*2,5-Dimethyl-4-aminophenylarsonic Acid*).—4.1 g. of the crude nitro acid was reduced in the usual way with 30 g. of ferrous sulfate in 90 cc. of water. The filtrate from the ferrous hydroxide was acidified with hydrochloric acid until congo red just began to change color and on rubbing the acid separated quickly as glistening scales. After filtering off and washing with ice water the yield was 3.0 g. A portion was recrystallized from water, forming thin, nacreous, serrated, almost colorless platelets containing one molecule of water of crystallization as described by Benda and Kahn,[2] who prepared the acid by the direct arsenation of *p*-xylidine. The amino acid is readily diazotizable and is sparingly soluble in cold water or alcohol, quite easily on boiling. When rapidly heated to 210°, then slowly, the anhydrous substance melts at 213–4° with decomposition.

Anhydrous subst.; Kjeldahl, 0.1526 g.; 6.35 cc. 0.1 *N* HCl.
Calc. for $C_8H_{12}O_3NAs$: N, 5.72. Found: 5.83.

3-Amino-4-hydroxyphenylarsonic Acid.—This substance, previously obtained by the reduction of 3-nitro-4-hydroxyphenylarsonic acid by the use of sodium hydrosulfite or sodium amalgam,[3] is also most conveniently prepared by the use of ferrous sulfate.

58.4 g. of 3-nitro-4-hydroxyphenylarsonic acid was reduced in the usual manner with 440 g. of ferrous sulfate. On acidifying the dark-

[1] *Ann.*, **320**, 339 (1902).
[2] Benda and Kahn, *Ber.*, **41**, 1676 (1908).
[3] D. R. P. 224,953; Ehrlich and Bertheim, *Ber.*, **45**, 757 (1911).

colored filtrate with acetic acid and rubbing, the aminohydroxyphenylarsonic acid separated in characteristic form. The yield was 42 g., or 80% of the theory. The recrystallized acid darkens and softens at about 220° and finally decomposes at about 290°.

Subst., 0.1525; Kjeldahl, 6.9 cc. 0.1 *N* HCl.
Calc. for $C_6H_8O_4NAs$: N, 6.01. Found: 6.34.

[Reprinted from The Journal of Biological Chemistry, September, 1918, Vol. xxxv, No. 3, pp. 497–508.]

THE INFLUENCE OF ELECTROLYTES UPON THE OSMOTIC PRESSURE OF GELATIN SOLUTIONS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 12, 1918.)

I.

It is generally stated in the literature of colloid chemistry that gelatin is influenced by both ions of a neutral salt, and that the influence of the anions of the salt is greater than that of the cation. The different anions are said to differ in their relative efficiency in a definite order, the now famous Hofmeister anion series. The writer has shown in a series of articles that all these statements are contrary to fact, and that the real facts are as follows: Common gelatin (*i.e.* gelatin near the point of neutrality) is affected only by the cations of a neutral salt, and not by the anions, univalent cations causing an increase in swelling, alcohol number, and viscosity, while bivalent cations cause no such change. The anion of the salt has no effect whatever.[1–5] The error into which the colloid chemists have fallen is due to the fact that they always investigated the effect of a neutral salt on a protein in the presence of the salt, while the writer took the precaution to wash the excess of salt away after it had time to act on the gelatin. In order to be able to do this he used gelatin in the form of a fine powder. A 1 per cent solution of the gelatin used for his experiments had a hydrogen ion concentration of 10^{-7}(pH = 7) (deter-

[1] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343.
[2] Loeb, *J. Biol. Chem.*, 1918, xxxiii, 531.
[3] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 77.
[4] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 395.
[5] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 489.

mined by Dr. Dernby). Since according to Michaelis[6] the isoelectric point of gelatin is at $C_H = 2.10^{-5}$ (pH = 4.7) the hydrogen ion concentration of the solution was less than that of the isoelectric point. In a paper which will appear in the first number of *The Journal of General Physiology* the writer will show that on the alkaline side from the isoelectric point gelatin (and amphoteric electrolytes in general) can exist in an ionized condition only as anions, and hence is capable of combining only with the cation of a neutral salt, as we had actually found. When the hydrogen ion concentration of the solution is greater than that corresponding to the isoelectric point, the gelatin (and amphoteric electrolytes in general) can exist in an ionized condition only as cations and hence can combine only with the anion of a neutral salt. Hence the statement that always both ions of a neutral salt act on the gelatin, and that the effect of the anion prevails in the case of gelatin is contrary to fact as well as theory.

In a paper generally accepted by colloid chemists Lillie[7] has claimed that while salts depress the osmotic pressure of gelatin solutions, acids and bases have the opposite effect; and he also expresses the general belief that both ions of a salt are effective and that the relative depressing effect of the anions of neutral salts follows the order of the Hofmeister anion series.

Lillie's conclusions are as follows:

"Acid and alkali increase the osmotic pressure of gelatin solutions; in general these substances affect the osmotic pressure of gelatin solutions in the same manner as they do the rate of swelling of solid gelatin plates immersed in water.

"Addition of salts depresses the osmotic pressure of both colloids [gelatin and albumin]; the degree of depression is a function of the nature of both the anion and the cation of the salt. It increases in the order: alkali metals < alkali earths < heavy metals (for cations); and CNS < I < Br < NO_3 < Cl < F < plurivalent anions—SO_4, tartrate, citrate, phosphate (for anions)."[8]

The statement that acid and alkali increase the osmotic pressure while salts depress it is as we shall show merely due to the

[6] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

[7] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

[8] Lillie, *Am. J. Physiol.*, 1907–08, xx, 169.

fact that Lillie worked with low concentrations of acids and bases in which the repressing effect of the presence of the electrolyte is less noticeable; while he worked with high concentrations of the neutral salts in which the repressing effect of the presence of the electrolyte was noticeable. Had he used stronger concentrations of alkalies and acids he would have noticed that the depressing effect which he observed in the case of salts is equally pronounced in the case of acids and bases. We shall show in this paper that the influence of electrolytes upon the osmotic pressure of gelatin runs parallel to the influence of the electrolytes upon the swelling, viscosity, and alcohol number of gelatin, demonstrated by our previous papers, inasmuch as only the cation of a neutral salt influences the osmotic pressure, while the anion is without influence upon common gelatin. We shall show, moreover, that while the salts with monovalent cation increase the osmotic pressure, the salts with bivalent cation have no such effect. The same difference exists between the influence of bases with univalent and bivalent cation and between monobasic and dibasic acids. In order to show this we have to wash away the excess of the electrolyte after it has had time to act on the gelatin.

The osmotic pressure was determined by the simple method devised by Lillie.[7] Small bags in the shape of Erlenmeyer flasks were made of collodion and the neck of each bag was fitted into a perforated rubber stopper and made tight with the aid of rubber bands. A vertical glass tube of about 2 mm. diameter went through the hole of the stopper into the gelatin solution which at the beginning filled the bag and the lower end of the glass tube to about 20 mm. All air bubbles were carefully removed from the bag. The latter was then put into a large beaker containing about 250 cc. of distilled water. The beakers were in a water bath kept at a temperature near 20°C. The final equilibrium of the column of liquid in the glass tube was reached over night and remained constant for the next 24 hours. Our final measurements were usually made after about 16 hours.

It was found that our collodion bags were easily permeable for salts like NaCl, but were practically impermeable for gelatin. When a bag was filled with a M/2 NaCl solution and put into distilled

water, the liquid in the bag or the glass tube serving as manometer rose during the first half hour to a height of about 70 mm. and then fell rapidly. In a few hours the osmotic pressure of the solution was zero. When the same experiment was made with a 1 per cent gelatin solution put into a beaker with distilled water, the pressure rose slowly to a height of about 80 mm. and remained there for several days—in fact as long as we continued the experiments. A test made of the distilled water surrounding the bag failed to give the biuret test after 16 hours.

Lillie's method may not be sufficiently accurate to satisfy the demands of the physicist,[9] but it is adequate to allow us to show that our former statements concerning the influence of neutral salts upon the physical properties of gelatin (swelling, viscosity, and resistance to precipitability by alcohol) hold also for the influence of neutral salts upon the osmotic pressure of gelatin solutions.

II.

On the basis of our previously published experiments we should expect that a treatment of powdered gelatin with a neutral salt with univalent cation should cause an increase in the osmotic pressure of the gelatin, after the excess of the salt solution is washed away, since the treatment of the gelatin with neutral salts should lead to the formation of metal gelatinates. The higher the concentration of the salt solution used, the greater the mass of metal gelatinate formed and the greater the increase in osmotic pressure to be expected. On the other hand, treatment of gelatin with neutral salts with bivalent metal should on the basis of our previously published experiments not cause any increase in osmotic pressure after the excess of salt has been washed away. Table I shows that these theoretical expectations are all fulfilled.

The method was the same as that used in the experiments on alcohol precipitability. 1 gm. of powdered gelatin going through a

[9] Two factors in our present method seem to cause a variation in the results; first, possible differences in the permeability of the membranes, and second, differences in the degree of washing away the electrolytes in solution and, if the washing is excessive, in combination with the gelatin.

50 mesh sieve but not through one of 60 was put for 1 hour into 100 cc. of the solution of a neutral salt to bring about a chemical reaction between gelatin and salt. The powder was then put on a filter, the salt solution allowed to drain off, and the gelatin was perfused six times with H_2O to remove the last traces of salt solution held in the capillary spaces or adhering to the surface of the granules. The gelatin was then heated for about 10 minutes in a water bath

TABLE I.

	Osmotic pressure in mm. of 1 per cent gelatin solution in distilled water, made from powdered gelatin treated for 1 hour with one of the following solutions and then perfused six times with 25 cc. H_2O to wash away the excess of salt solution.												
	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
NaCl	134	122	123	107	99	93	81	80	77	77	71	74	77
NaBr	136	133	123	118	107	88	78	79	82	78	76	78	79
NaCNS		132		118	107	94	94	82	88	78	75	73	74
Na acetate	170	151	139	127	108	92	89	80	79	78	77	75	81
KCl	116	120	116	100	95	80	72	75	68				70
$LiNO_3$	121	120	104	102	91	84	81	73	73				73
NH_4Cl	145	138	137	127	105	94	86	80	83	81	77	77	77
Na_2SO_4	159	166	156	151	136	107	102	83	80				73
Na_2 tartrate	166	159	142	129	116	110	98	77	60	73	78		72
Na_2 succinate		170	163	144		132	112	91	83	86	81		84
Na_2 oxalate		100	91	88	90	94	90	88	81	78	82	81	
Na_2 malate		166	152	142	135	121	102	89	76	79	77	73	
$MgCl_2$		79	83	82	82	77	78	81	81	75	78	78	80
$CaCl_2$	77	76	82	76	84	84	83	80	81				80
$SrCl_2$	75	81	78	80	83	81	82	82	76				78
$BaCl_2$		69	70	73	76	76	75	80	82	81	81	80	82
$MgSO_4$	84	80	80	82	83	82	79	81	80	73	82	82	81

of 50°C. so as to liquefy the gelatin, and distilled water was added to make the solution 1 per cent. With this gelatin solution free from the salt with which the gelatin had been treated the influence of salts upon the osmotic pressure was determined.

In looking at the results in Table I it is strikingly clear, first, that a treatment of the gelatin with a neutral salt with a bivalent

metal, $MgCl_2$, $CaCl_2$, etc., leads to no increase in osmotic pressure, even if we go up to M/4 solutions. (We cannot go beyond this limit on account of the liquefying effect of the higher concentrations upon the gelatin and the difficulty of freeing the solution from the excess of salt.)

Second, it is obvious that $MgSO_4$ acts exactly as $MgCl_2$, thus indicating that the anion has no influence.

Third, it is obvious that the salts with univalent cation increase the osmotic pressure of the gelatin.

Fourth, it is obvious that the molecular concentration required to cause a perceptible rise (to 90 mm. or above) in the osmotic pressure is for salts of the type NaCl (univalent cation, univalent anion), higher than for salts of the type Na_2SO_4 (univalent cation and bivalent anion). For the former it is between M/128 and M/256, and for the latter between M/256 and M/512; indicating that the concentration of the cation alone (or practically exclusively) determines the effect of the salt regardless of the nature of the anion, though this latter result does not come out as strikingly as in the measurements of the precipitability of gelatin with alcohol or the limiting concentration for additional swelling.[2, 3] Within the limits of the imperfections of our present osmotic pressure determinations the results on osmotic pressure confirm our theory of the action of neutral salts upon proteins expressed in our former papers. The only exception noticed is the oxalate solution, which apparently caused a precipitate in the gelatin.

III.

Our theory furthermore demands that bases should influence the osmotic pressure of gelatin in a sense parallel to that of the corresponding neutral salts; namely, a previous treatment of powdered gelatin with NaOH and KOH should cause a considerable increase in osmotic pressure (after the free base is washed away) while $Ba(OH)_2$ and $Ca(OH)_2$ should cause no or at the best only a slight increase in osmotic pressure under the same conditions of experimentation. According to our theory bases act upon proteins of the type of gelatin (provided that pH > 4.7) like neutral salts, both causing the forma-

tion of metal proteinates, while the anion of the salt has apparently no influence upon the protein. A glance at Table II shows this expectation to be correct. 1 gm. of powdered gelatin was put for half an hour into the solutions of the different bases and then treated in a similar way as in the case with salts. The excess of alkalies was allowed to drain off and the gelatin was washed four times with 25 cc. of H_2O. A treatment of the gelatin with $Ca(OH)_2$ leads to no increase in the osmotic pressure of the solution, while a treatment with $Ba(OH)_2$ may lead to a slight increase after the excess of base has been washed away. On the other hand, treatment of the gelatin with NaOH and KOH leads to a considerable increase in the osmotic pressure after the excess of alkali has been washed away.

TABLE II.

	Osmotic pressure in mm. of 1 per cent gelatin solution in distilled water, made from powdered gelatin treated for 30 min. with 100 cc. of one of the following solutions and then perfused four times with 25 cc. H_2O to wash away the excess of salt solution.										
	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
NaOH.....	172		165	129	115	102	98	90	88	90	76
KOH.....	154	155	147	120	98	96	95	88	87	79	77
$Ba(OH)_2$.....		77	95	95	97	99	95	90	89	88	
$Ca(OH)_2$.....			85	89	92	87					78

IV.

The fact that salts or bases with bivalent cation do not lead to an increase in the osmotic pressure (or the swelling, the viscosity, and resistance to alcohol precipitation) of proteins might be interpreted to mean that such salts or bases do not combine with proteins. Such a view is, however, repudiated by the facts of antagonistic salt action, inasmuch as it can be shown that the addition of a comparatively small amount of a salt with bivalent cation inhibits the increase in osmotic pressure (and also the increase in swelling, viscosity, and resistance to alcohol precipitation)[1–4] caused by a treatment of the proteins with a salt or base with univalent cation after the excess of salt solution is washed away.

The method of our experiment was as follows: 1 gm. of powdered gelatin was put for 1 hour into 100 cc. M/8 NaCl containing 0, 1, 2, 4, 6, 8, 12, 16, and 32 cc. M/8 $CaCl_2$ or $BaCl_2$. After 1 hour the gelatin was put into a funnel, the salt solution was allowed to drain off, and the powder was then perfused six times with 25 cc. of H_2O to remove the last traces of the salt solution. After this, 1 per cent solutions of these different gelatins in distilled water were made, and the osmotic pressures were determined. It was found that the addition of Ca or Ba diminishes the rise in osmotic pressure which is caused by the pure solution of NaCl, and this diminution is the greater the more Ca or Ba had been added. The addition of 1 or 2 cc. of $CaCl_2$ or $BaCl_2$ had already a noticeable effect (Table III).

TABLE III.

	Osmotic pressure in mm. of 1 per cent gelatin solution in distilled water made from powdered gelatin treated for 1 hour at 20° with 100 cc. of an M/8 NaCl solution containing various amounts of M/8 $CaCl_2$ or $BaCl_2$ and then perfused six times with 25 cc. H_2O to wash away excess of salt.									
100 cc. M/8 NaCl + cc. $CaCl_2$ or $BaCl_2$	0	1	2	4	6	8	12	16	24	32
NaCl + $CaCl_2$	131	129	120	107	106	99	94	90	85	84
NaCl + $BaCl_2$	134	125	111	98	110	29	97	83	86	74

This proves that the salts with bivalent cation prevent the increase of osmotic pressure caused by the salts with univalent cation. The same antagonism has already been demonstrated in the writer's previous papers for additional swelling, increase in viscosity, and resistance to alcohol precipitation. The reader will remember that in all these experiments the excess of salt solution was washed away after the salt had had time to act on the gelatin. These experiments prove that the salts with bivalent cations react with gelatin.

According to our theory the same antagonism as between NaCl and $BaCl_2$ should exist between NaOH and $Ba(OH)_2$. This was found to be true.

Powdered gelatin was put into 100 cc. of M/32 NaOH (free from carbonate) containing 0, 1, 2, 4, 6, 12, 16, 24, and 32 cc. M/32 $Ba(OH)_2$. The mixture was contained in tightly stoppered bottles,

and the powder remained in the mixture 30 minutes. After this the gelatin was put into funnels, the alkali solution allowed to drain off, and the gelatin was perfused four times with 25 cc. of H_2O to remove the last traces of the original solution from the capillary spaces between the granules. Table IV shows that the expected antagonism between NaOH and $Ba(OH)_2$ was found, and in addition that the antagonistic action exists also for swelling and precipitation with alcohol.

These experiments are only intelligible on the assumption that the salts and bases with bivalent cation influence the protein in the same way and that, moreover, the bivalent cations inhibit the effect of the salts and bases with univalent cation.

TABLE IV.

	1 gm. powdered gelatin put for 30 min. into one of the following solutions and then perfused four times with 25 cc. H_2O.								
100 cc. M/32 NaOH + cc. M/32 $Ba(OH)_2$	0	1	2	4	8	12	16	24	32
Osmotic pressure in mm. of 1 per cent gelatin solution	185	147	148	147	133	126	115	109	105
Total swelling in mm. of height of cylindrical mass of gelatin	70	71	70	61	50.5	51	46	45	31
Cc. 95 per cent alcohol required to precipitate 5 cc. of 1 per cent gelatin solution	∞	∞	∞	∞	∞	14.9	11	10.4	7.1

V.

We will now show that if we do not remove the excess of solution of electrolyte with which the gelatin is treated, the effects of the electrolyte will be repressed and that this repression varies with the concentration and nature of the electrolyte. The measurements for the effect of electrolyte upon the osmotic pressure of gelatin will thus be the resultant of opposite influences of the electrolyte, the relative quantity of which is unknown in each case; and what appears to be the effect of the electrolyte upon the gelatin is in reality the difference between the unknown effect of the electro-

lyte upon the gelatin and the equally unknown repressing effect of the presence of the excess of electrolyte. Instead of arriving at the clear results which our method gives, we arrive at indefinite results which misled Hofmeister into the erroneous statement concerning the effect of anions upon the swelling;[10] and the authority of Hofmeister obviously induced Lillie to look for a confirmation of Hofmeister's statement.

TABLE V.

	Osmotic pressure in mm. of 1 per cent gelatin solution in presence of electrolyte.													
	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	M/16384	H_2O
I.														
HNO_3	47	48	58	46	104	169	286	352	330	205	81	26		
NaOH			71	70	85	124	122	213	206	185	134	112		90
NaCl		37	33	38	35	41	42	63	74	83	90	84		88
II.														
H_2SO_4				39	48	62	90	121	93	140	103	34	46	81
$Ba(OH)_2$					60	49	50	71	102	107	115	110		
$BaCl_2$		35	32	30	31	33	33	32	38	42	60	73		
III.														
$NaNO_3$		32	34	28	38	39	50	61	62	81	78	91		87
LiBr		35	35	42	42	39	58	62	71	70	89	87		
Na acetate		43	43	45	Lost.	52	66	70	80	101	118	110		91
NaCNS			43		55	56	60		85	95	100	100		102
Na_2 tartrate			40	37	38	41	62	56	60		100	105	103	
Na_2SO_4		27	25	41	46	35	41	47	50	62	92	80		90

The experiments represented in Table V were made in a similar way as those of Lillie. A 1 per cent solution of gelatin was made up in the solution of the electrolyte whose effect was to be studied, and the same solution of the electrolyte was put into the beaker surrounding the collodion bag containing the 1 per cent gelatin solution. Since the membrane is entirely permeable to crystalloids, the osmotic pressure of the gelatin solution can be measured in this way.

[10] Hofmeister, F., *Arch. exp. Path. u. Pharmakol.*, 1891, xxviii, 210.

Group I, Table V, gives the osmotic pressure of a 1 per cent gelatin solution in the presence of a monobasic acid, HNO_3, a base with univalent cation, NaOH, and a neutral salt, NaCl. It is obvious that the presence of the base NaOH depresses the osmotic pressure below that of a non-treated gelatin solution as soon as the concentration of NaOH is M/64 or above; and the same is true as soon as the concentration of the acid is M/32 or above. According to Table I NaCl only begins to cause a marked increase in osmotic pressure in concentrations above M/128 or M/64, but the presence of the salt in these or higher concentrations prevents the manifestation of this increase.

Hence the behavior of gelatin in the presence of electrolytes conforms with the results and conclusions from our former experiments.

Furthermore, a glance at Group II, Table V, brings out a further confirmation of our theory; namely, that neutral salts with bivalent cation, $BaCl_2$, behave like bases with bivalent cation, $Ba(OH)_2$, and acids with bivalent anion, H_2SO_4, inasmuch as in all three cases the osmotic pressures run lower than the corresponding pressures in Group I, which was to be expected according to our previous publications.

Group III, containing different salts of Na, shows that there is no basis for assuming the validity of the Hofmeister series upon the osmotic pressure. The variations between the effects of sodium salts with different anions are within the limits of error of the method or due to differences in the repressing effect of the different salts present. Neither do Lillie's own observations support the validity of the Hofmeister series. He states:[11] "Individual variability is so strongly marked in colloidal solutions that little reliance can be placed on the order of action observed in a single series of determinations. . . . ." When we free the solution from an excess of salt, the results become clear and leave no doubt that the alleged anion effect of neutral salts upon gelatin does not exist.

SUMMARY.

1. It is generally stated in colloid chemistry that only acids and bases cause an increase in the swelling, the viscosity, and the osmotic

[11] Lillie, *Am. J. Physiol.*, 1907–08, xx, 152.

pressure of hydrophilic colloids, while neutral salts of the type of NaCl have little or, according to Lillie, a directly opposite effect. The writer has found that neutral salts act exactly like acids and bases inasmuch as neutral salts and bases with univalent cation and acids with univalent anion increase the swelling, the viscosity, and resistance to precipitation by alcohol of gelatin, while salts and bases with bivalent cation and acids with bivalent anion (with the exception of some organic acids) have no or only a slight effect. In this paper it is shown that the same is true for the influence of electrolytes upon the osmotic pressure of gelatin.

2. It was found by the writer that only the cation of neutral salts acts upon gelatin in neutral solutions or in solutions with pH > 4.7, while the anion of neutral salts does not influence the osmotic pressure and the other physical qualities of gelatin (swelling, viscosity, and alcohol precipitability).

3. These experiments contradict the statements generally made in colloid chemistry that the influence of neutral salts upon a protein is the algebraic sum of the opposite effects of the oppositely charged ions of the neutral salts. They also contradict the statement that common gelatin (at the point of neutrality) is influenced more strongly by the anion than by the cation of a neutral salt.

4. The difference between our results and those of the older experimenters is due to the fact that in the older experiments the effects of neutral salts upon gelatin were always measured in the presence of the salts. We were able to show that the presence of the salt represses the manifestation of its effects upon the gelatin and that it is therefore necessary to remove the excess of the salt after it has had time to act on the protein.

5. Our results are in agreement with a theory of the behavior of amphoteric electrolytes, which will appear in *The Journal of General Physiology;* namely, that on the more acid side of their isoelectric point amphoteric electrolytes can exist, when ionized, only in the form of cations, while on the less acid side of their isoelectric point they can exist, when ionized, only as anions. In the former case the ampholyte can combine only with the anion, in the latter only with the cation of a neutral salt. Since gelatin has its isoelectric point at pH = 4.7, while the gelatin used by us had a pH = 7, such gelatin could react only with the cation of neutral salts.

[Reprinted from The Journal of General Physiology, September 20, 1918, Vol. i, No. 1, pp. 39–60.]

AMPHOTERIC COLLOIDS.

I. Chemical Influence of the Hydrogen Ion Concentration.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 26, 1918.)

I.

It is often stated (Pauli, Höber, and others) that both ions of a neutral salt affect the physical properties of an amphoteric electrolyte (ampholyte) simultaneously and in the opposite sense; so that the total result is the algebraic sum of the opposite action of the oppositely charged ions of the neutral salt.[1] The writer has been able to show by experiments on gelatin and on pig's bladder that this statement is not correct, but that apparently only one of the two ions of the neutral salt acts upon the ampholyte; namely, the one which has the opposite electric charge from that of the ampholyte.[2]

The hydrogen ion concentration of the gelatin solution used by the writer was 10^{-7} (or in Sörensen's logarithmic symbol pH = 7.0) and this gelatin reacted with neutral salts as if it were an anion capable of combining with the cation of a neutral salt. When, however, powdered gelatin is treated for some time with an acid, *e.g.* HCl, gelatin chloride (or hydrochloride) is formed, which is supposed to dissociate electrolytically into a positive gelatin ion and a negative chlorion. The writer found that when the supernatant

[1] This and other erroneous statements current in colloid chemistry are due to the fact that the previous investigators always studied the action of neutral salts upon proteins in the presence of the salt. The writer removed these salts after they had a chance to act on the gelatin. This procedure is necessary on account of the fact that the manifestation of the effect of the electrolyte upon the gelatin is repressed in the presence of the electrolyte.

[2] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343; 1918, xxxiii, 531; xxxiv, 77, 395, 489; 1918, xxxv, 497.

acid is washed away from such powdered gelatin it is affected only by the anion of a neutral salt, while the cation of the salt used has apparently no effect.

The writer found that when powdered gelatin is treated for some time with a base, *e.g.* NaOH, the gelatin (after the supernatant NaOH is washed away) is only capable of combining with the cations of neutral salts, while the anions of neutral salts are without effect.

It seemed necessary to find out where the turning point for these two different modes of reaction lies, whether at the point of neutrality of the solution or at the isoelectric point of gelatin. The fact that gelatin solutions at the point of neutrality (pH = 7.0) react with neutral salts as if they had previously received an alkaline treatment, suggests that the turning point lies at a more acid reaction of the gelatin and since the isoelectric point lies at a more acid reaction it was *a priori* likely that the isoelectric point for gelatin might be the turning point.

Amphoteric electrolytes are defined by the fact that they can dissociate electrolytically as acids as well as bases. When they dissociate as acids they are supposed to send more H ions than OH ions into solution and the ampholyte ion migrates in the electric field to the anode. When they dissociate as bases they are supposed to send more OH ions than H ions into solution, and the ampholyte migrates in the electric field as a cation. At the isoelectric point the ampholyte is supposed to send out an equal number of H and OH ions and it migrates in neither direction. Gelatin is naturally a stronger acid than base and hence migrates to the anode. By increasing the hydrogen ion concentration of the surrounding solution through the addition of some acid the dissociation of gelatin as acid can be repressed to the level of its dissociation as a base. This is the isoelectric point at which it migrates in neither direction and at which the total number of ions it sends into a solution is a minimum. With the addition of more acid it migrates to the cathode. Michaelis determined the isoelectric point of gelatin solutions by the migration method and found it in the neighborhood of $C_H = 2.10^{-5}$ (or in Sörensen's logarithmic symbol pH = 4.7).[3] Since the solution of gelatin used had a pH of 7.0 it was on the alkali side of the isoelectric point. We shall

[3] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

show in this paper that gelatin on the alkali or less acid side of its isoelectric point can combine only with the cations of neutral salts, as we had actually found empirically in our previously published papers.

Moreover, it will follow from the experiments to be published in this paper that the gelatin ion can dissociate electrolytically only as one of the two, either as a cation or as an anion but not as both simultaneously (except to a negligible degree), and that the hydrogen ion concentration of the solution alone determines in which of the two ways it dissociates. We shall also show that the isoelectric point is not only the point where the gelatin sends out as many H as OH ions but that it is the point where it apparently sends out neither; *i.e.*, where it cannot exist in an ionized condition at all.

II.

Our method of testing whether gelatin reacts with the cation or the anion of a neutral salt is based on the difference of the effects of univalent and bivalent ions upon gelatin. It was found in the writer's experiments on osmotic pressure, viscosity, alcohol precipitation, and swelling that a salt of the type Na_2SO_4 (univalent cation, bivalent anion) of a certain molecular concentration, *e.g.* M/128, has quantitatively and qualitatively the same effect upon gelatin (pH = 7.0) as a salt of the type NaCl at the molecular concentration M/64; *i.e.*, twice the molecular concentration of the Na_2SO_4.[4] This proves that only the cation of the salt and not the anion acts upon the gelatin. It was found, moreover, that all neutral salts with monovalent cation (Li, Na, K, NH_4) cause an increase in osmotic pressure, viscosity, alcohol number, and swelling of gelatin, while salts with a bivalent cation (Mg, Ca, Sr, Ba, Co, Mn) cause no such increase; and if a salt with bivalent cation is added in small quantity to a large quantity of a salt with univalent cation, the increase in osmotic pressure, viscosity, alcohol number, and swelling caused by the salt with univalent cation is inhibited (antagonistic salt action).[5] The same is

[4] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 77, 489.

[5] All these effects were measured after the excess of the electrolyte was washed away (see footnote 1).

true for gelatin previously treated with NaOH. When such gelatin is subsequently treated with a salt with univalent cation its viscosity, osmotic pressure, swelling, etc., are increased, while when it is treated with a salt with bivalent cation this increase does not occur and its previous osmotic pressure, etc., are diminished. In all these cases the gelatin migrates as an anion, and the compounds formed are metal gelatinates where the metal is that of the alkali or salt used.

On the other hand, if gelatin has first been treated with an acid, *e.g.* HCl, gelatin chloride is formed which shows an increase in osmotic pressure, swelling, viscosity, and alcohol number. That such a gelatin (which is freed from the original acid by washing) reacts only with the anion of neutral salts is proved by the fact that a solution of a salt of the type $Ca(NO_3)_2$ (univalent anion, bivalent cation) influences gelatin chloride quantitatively and qualitatively like a salt of the type $NaNO_3$ (univalent anion, univalent cation) of half the molecular concentration; *e.g.*, M/128 $Ca(NO_3)_2$ acts like M/64 $NaNO_3$.[4] This is only possible if the anions of the neutral salt react with the gelatin chloride, while the cations have no effect, since otherwise the depressing effect of Ca mentioned before should be noticeable, which is not the case. If gelatin chloride freed from the excess of acid by washing is treated with a sufficiently high concentration of a neutral salt with univalent anion the osmotic pressure, swelling, viscosity, etc., of the gelatin increase after the salt is washed away. If, however, the anion of the salt used is bivalent, *e.g.* sulfate, oxalate, succinate, it causes less increase or an actual decrease of the osmotic pressure, viscosity, alcohol number, and swelling of the gelatin.

Hence by making use of the depressing effects of bivalent ions and the opposite effect of the univalent ions we can determine at which pH the gelatin begins to combine with the cation and ceases to combine with the anion of a neutral salt. Our experiments show that this critical point lies for gelatin at a hydrogen ion concentration 2.10^{-5} (pH = 4.7), *i.e.* the isoelectric point of gelatin, and not at the point of neutrality.

Our method was the same as in our previous experiments. 1 gm. of finely powdered gelatin was put into each of a series of beakers containing 100 cc. of a solution of HCl of various concentrations (M/8 to M/8192). This series served as a control. We then experimented with HCl solutions of different concentrations (M/8 to M/8192), each containing a certain salt (*e.g.* Na_2SO_4 or $Ca(NO_3)_2$) in the same concentration (M/16). The gelatin remained for 30 minutes in these solutions of a temperature of 20°C. The gelatin was then poured into cylindrical funnels of the same diameter, to allow the acid or acid-salt mixture to drain off, and the gelatin was then perfused three times with 25 cc. of distilled water of 10°C., and a fourth time with H_2O of 20°C.[6] The gelatin was then melted and made into a 1 per cent solution. Dialysis experiments, titration experiments, and determinations of pH by Sörensen and Clark's indicator method[7] made it probable that the solutions contained no acid except that due to hydrolytic dissociation of the gelatin salt formed; it was also possible to show that the neutral salt added to the HCl solution had been washed away.

The following physical qualities of the washed gelatin were ascertained:

1. The conductivity $\left(\frac{10{,}000}{\text{ohms}}\right)$, at 24°C.

2. The osmotic pressure expressed in mm. height of the 1 per cent gelatin solution in the manometer tube.

3. The total swelling expressed in mm. of the height of the cylindrical mass of gelatin.

4. The alcohol number; *i.e.*, the cc. of 95 per cent alcohol required to precipitate 5 cc. of 1 per cent gelatin solution at 20°C.[5]

The results are given in the form of curves in which the logarithms of the concentration of the acid used are the abscissæ and the above mentioned values for conductivity, osmotic pressure, swelling, and alcohol number, the ordinates. The pH of each gelatin solution was

[6] The water was used at these temperatures to prevent excessive swelling and a retardation of the process of filtration.

[7] We are obliged to Dr. Dernby for demonstrating to us the use of these methods.

[5] The viscosity of the solutions was determined in a special set of experiments and the viscosity curves ran parallel to those for osmotic pressure. They may therefore be omitted in this paper.

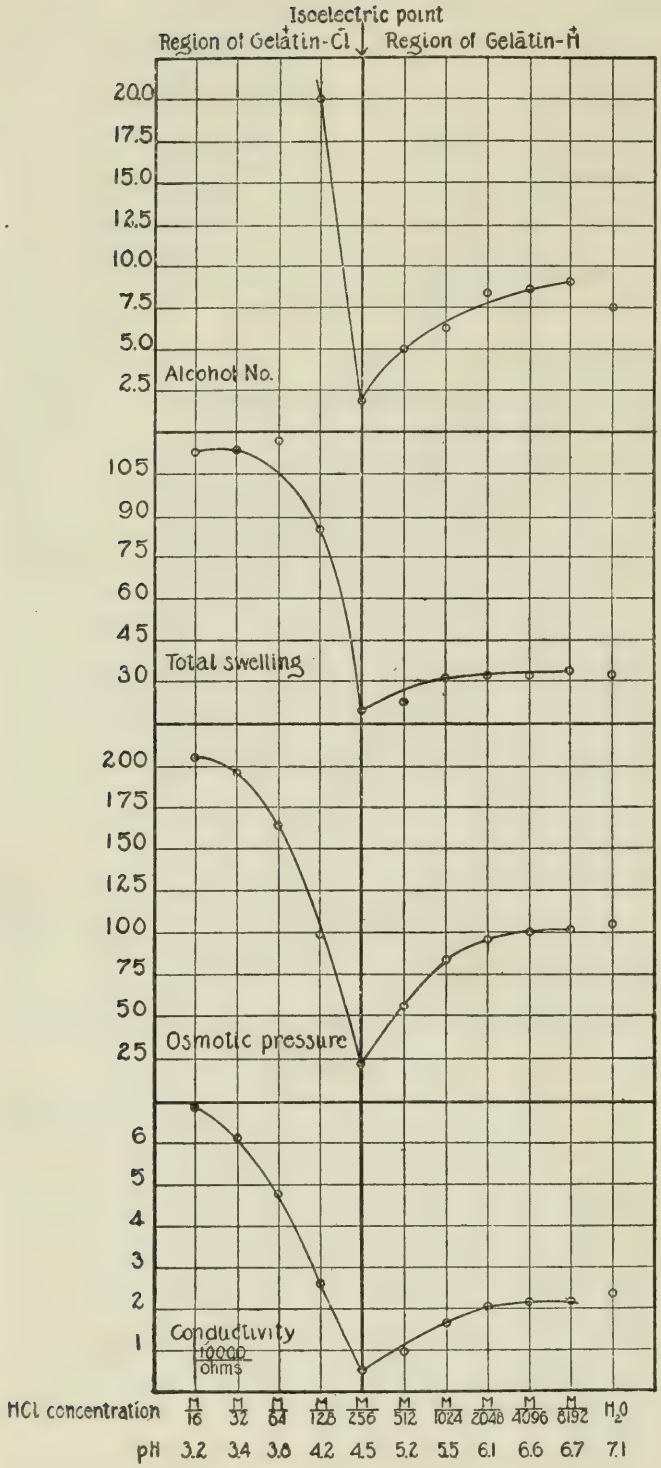


FIG. 1. HCl curves. Curves of the conductivity, osmotic pressure, swelling, and alcohol number of gelatin previously treated with various concentrations of HCl and then freed from excess of HCl by washing with water. On the axis of abscissæ are the logarithms of the HCl concentration used, and under each concentration is the pH of the 1 per cent gelatin solution in H_2O (made from the washed gelatin). The ordinates are the values for conductivity, osmotic pressure, swelling, and alcohol number for the gelatin. In the region of the isoelectric point (pH = 4.7) all the curves have a minimum. On the left of this point gelatin exists in the form of gelatin chloride (with high ionization), the curves rising more rapidly than on the right of the isoelectric point where the gelatin exists in the form of common gelatin which dissociates as a very weak acid. All four curves are nearly parallel.

ascertained and is put under the figures for the logarithms of the concentration.

We now will in turn discuss the four main groups of curves.

Fig. 1 gives the curves for gelatin treated for 30 minutes with HCl alone and then freed from the supernatant and intercapillary acid (with the exception of that formed by hydrolysis of gelatin chloride). The striking fact is that the conductivity, osmotic pressure, swelling, and alcohol number (and viscosity, though this curve is omitted) have all a minimum near a point where the hydrogen ion concentration pH = 4.7; *i.e.*, at about the isoelectric point as determined for gelatin solution by Michaelis on the basis of migration experiments. We may add that the transparency of the solution was also a minimum at the isoelectric point, where the solution was generally quite opaque after standing over night.

Though it was already known[3] that the viscosity and the swelling of gelatin are a minimum at pH = 4.7, our experiments for the first time furnish the proof that this is also the exact location of the minimum for conductivity, osmotic pressure, alcohol number, and transparency.

The second fact of significance is the sudden rise of the system of curves on either side of the isoelectric point. Since the conductivity curve is the direct expression of the degree of electrolytic dissociation existing in the gelatin solution (which had been freed from all ions except those formed by electrolytic and hydrolytic dissociation of the gelatin salt), these curves may be considered as a function of the degree of electrolytic dissociation of the gelatin.

Gelatin may exist in the form of non-ionized gelatin on both sides of the isoelectric point, but we shall endeavor to show that ionized gelatin exists in a different ionic state on the two sides of the isoelectric point. On the right, less acid side of the isoelectric point it can only exist as a negative ion (*e.g.* $\text{gelatin-H}^{-}{}^{+}$ or $\text{gelatin-Na}^{-}{}^{+}$, etc.), while on the left, more acid side of the isoelectric point it can only exist as a cation (*e.g.* $\text{gelatin-Cl}^{+}{}^{-}$ or $\text{gelatin-OH}^{+}{}^{-}$). When we study the curves for gelatin treated previously with HCl and freed from the excess of HCl by washing as described, the two branches of the curves on the two sides of the isoelectric point are asymmetrical (Fig. 1).

The left branches rise higher than the right ones on account of the fact that on the left side of the isoelectric point we have gelatin chloride which as a salt has a high dissociation constant and which dissociates into $\overset{+}{\text{gelatin}}$ and $\overset{-}{\text{Cl}}$, while on the right of the isoelectric point the curves are the expression of the electrolytic dissociation of common gelatin which dissociates like a very weak acid into $\overset{+}{\text{gelatin}}$ and $\overset{-}{\text{H}}$. Hence the limited rise of conductivity on the right and the considerable rise on the left of the isoelectric point.

The parallelism between the curve for conductivity and that for the other physical properties of the gelatin solution is so complete that the idea is unavoidable that the degree of conductivity, *i.e.* of the ionization of the gelatin, determines all the other physical properties of the gelatin solution; the pH only entering to the extent as it influences the possibility and character of the ionization of the gelatin. We shall see presently, however, that this parallelism between the conductivity and the curve for the other physical properties of the gelatin solution does not hold throughout but that in addition to the degree of electrolytic (and hydrolytic) dissociation still another variable enters. The main fact for us is the typical asymmetry of the two branches of the curve to the left and to the right of the isoelectric point, the branch on the left (the more acid side) being steeper and much higher than the branch on the right.

A comparison of the curves of Figs. 2, 3, and 4 with those of Fig. 1 will prove that gelatin when it ionizes will ionize on the right, less acid side of the isoelectric point as an anion, on the left, more acid side of the isoelectric point as a cation.

Fig. 2 represents the effect of the same concentrations of HCl as Fig. 1, except that each acid solution contained so much Na_2SO_4 as to make them all M/16 for this salt. We will call this system of curves the Na_2SO_4-HCl curves to distinguish them from the pure HCl curves represented by Fig. 1. The powdered gelatin was put for 30 minutes at 20° into these acid-salt mixtures and then freed from all the supernatant free acid and salt by the process of washing described in the writer's previous papers. If it is true that the isoelectric point is also a chemical turning point and that gelatin can ionize only as an

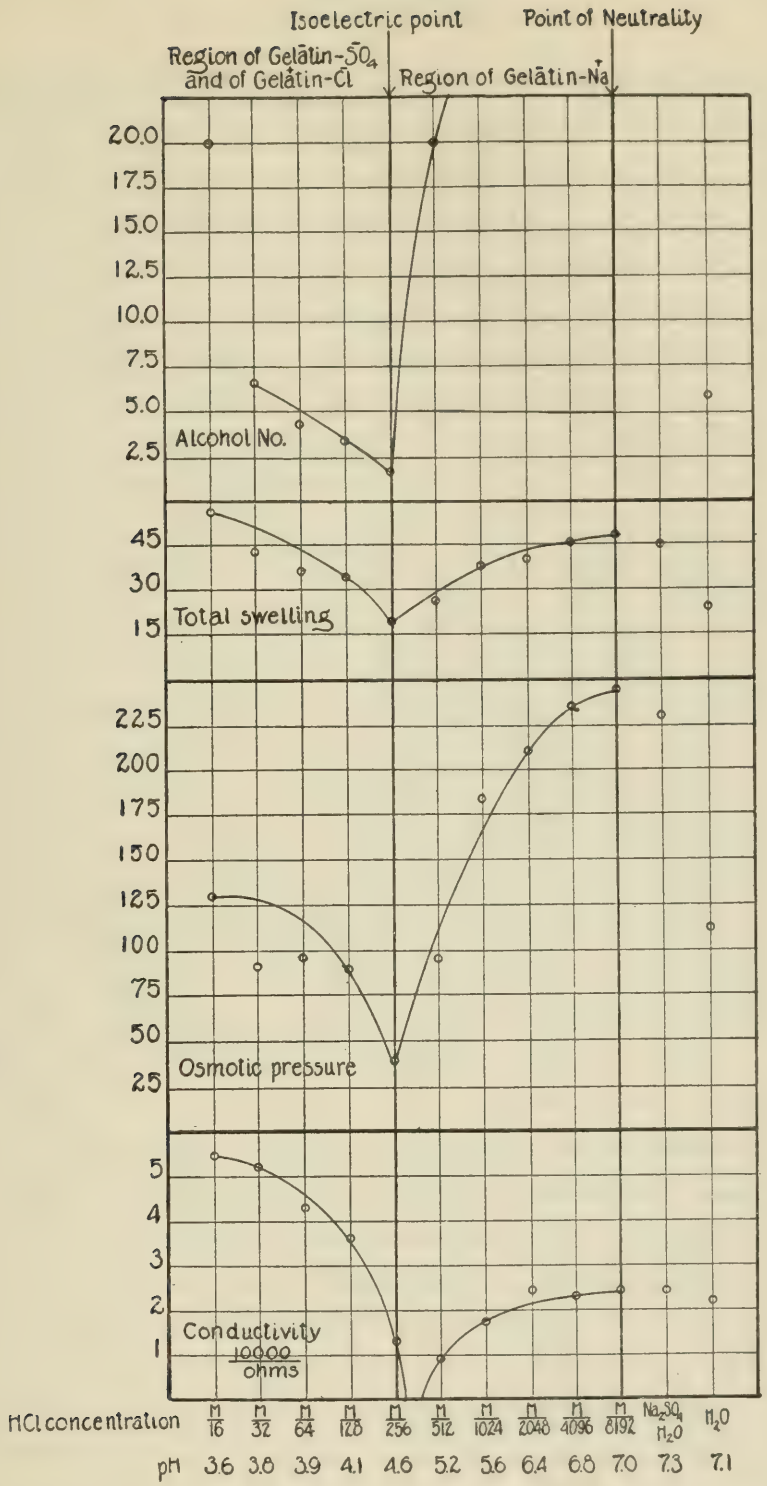


FIG. 2. Na_2SO_4-HCl curves. Isoelectric point and minimum again near pH = 4.7. Values for osmotic pressure, swelling, and alcohol number on right of isoelectric point, where gelatin exists as sodium gelatinate, higher than on left, where it exists as mixture of gelatin sulfate and gelatin chloride. The curves for osmotic pressure, swelling, and alcohol number are *not* parallel to the curve for conductivity.

anion on the right side of this point, and as a cation on the left side, we should be able to predict the difference between the two systems of curves, namely the HCl curves and the Na_2SO_4-HCl curves (Figs. 1 and 2), as far as osmotic pressure, swelling, and alcohol number are concerned. On the right, less acid side of the isoelectric point gelatin can dissociate only as a negative ion and hence, reacting with Na_2SO_4, it should be transformed into sodium gelatinate. On the left side, where gelatin can only exist as cation, in a mixture of HCl and Na_2SO_4 gelatin chloride and gelatin sulfate should be formed. In the Na_2SO_4-HCl curves (Fig. 2) we should, therefore, expect to find on the right side of the isoelectric point (pH > 4.7) sodium gelatinate, showing a high osmotic pressure, a high alcohol number, etc. On the left side of the isoelectric point where gelatin exists as gelatin chloride and gelatin sulfate, we should find lower values for osmotic pressure, alcohol number, etc., since we have shown in former experiments that the addition of SO_4 to Cl represses the increase in osmotic pressure, viscosity, swelling, caused by Cl. Hence we should expect an asymmetry in this system of curves of exactly the reverse kind of that found in the pure HCl experiment. A glance at Fig. 2 shows that this is the case, for osmotic pressure, alcohol number, but not for conductivity and pH, which are the same for the same HCl concentrations as in the pure HCl experiment (Fig. 1). We shall return later to this difference which is of great theoretical importance.

Hence we reach the conclusion that the isoelectric point of the gelatin is also the turning point for the chemical reaction between gelatin and neutral salt, inasmuch as the gelatin on the less acid, right side of the isoelectric point can exist in an ionized state only as a negative gelatin ion capable of combining with cations but not with anions, while on the left (more acid) side of the isoelectric point it can exist in an ionized state only as a positive gelatin ion capable of combining with the anions of a neutral salt.

This conclusion is confirmed by the experiments of the $Ca(NO_3)_2$-HCl series. In this series, represented by the curves of Fig. 3, the same concentrations of HCl from M/8 to M/4096 were used and each HCl solution contained so much $Ca(NO_3)_2$ as to make it M/16 in regard to $Ca(NO_3)_2$. If our theory is correct, the asymmetry of the

curves for osmotic pressure, swelling, and alcohol number should be the reverse for the $Ca(NO_3)_2$ series as that for Na_2SO_4. On the right side of the isoelectric point (for pH > 4.7) gelatin should exist in the form of Ca gelatinate which according to our previous observations has a low osmotic pressure, a low alcohol number, low viscosity, etc. On the left side of the isoelectric point it should exist in the form of gelatin chloride and nitrate in which form gelatin has a high osmotic pressure, swells considerably, has a high viscosity, and a high alcohol number. The forms of the curves actually found are represented in Fig. 3, and show that the facts agree with our theory.

Our theory finally demands that the two branches of the curve should become almost symmetrical in regard to the isoelectric point, if we add to the HCl a salt with univalent cation and univalent anion, *e.g.* NaBr. NaBr was added to the HCl solutions so as to make the solution M/16 in regard to this salt, and Fig. 4 gives the curves for gelatin treated with NaBr-HCl. It is obvious that the branches of the curves to both sides of the isoelectric point are much more symmetrical than was the case in the three other groups of curves. In this case we have on the right side of the isoelectric point Na-gelatinate, on the left a mixture of gelatin bromide and chloride. On both sides of the isoelectric point we have gelatin in combination with univalent ions, which as we have shown in our previous papers yield a high osmotic pressure, a high swelling, etc.

The experiments were repeated with other salts, $Mg(NO_3)_2$, Na_2 oxalate, etc., and our conclusions were confirmed. In the experiments described in this paper the reaction between the anions of the neutral salt with gelatin took place while the gelatin was in the acid solution; while the reaction between the cations of the neutral salt and gelatin could not take place while the gelatin was in the acid solution since all these concentrations were on the acid side of the isoelectric point of gelatin. This reaction must have taken place after the acid treatment and during the process of washing with H_2O when the gelatin previously treated with M/256 HCl had reached a pH > 4.7.

This conclusion is confirmed by the experiments in which the gelatin was first treated with acid, then freed from the excess of acid by perfusions with distilled water, and was then treated with a salt and

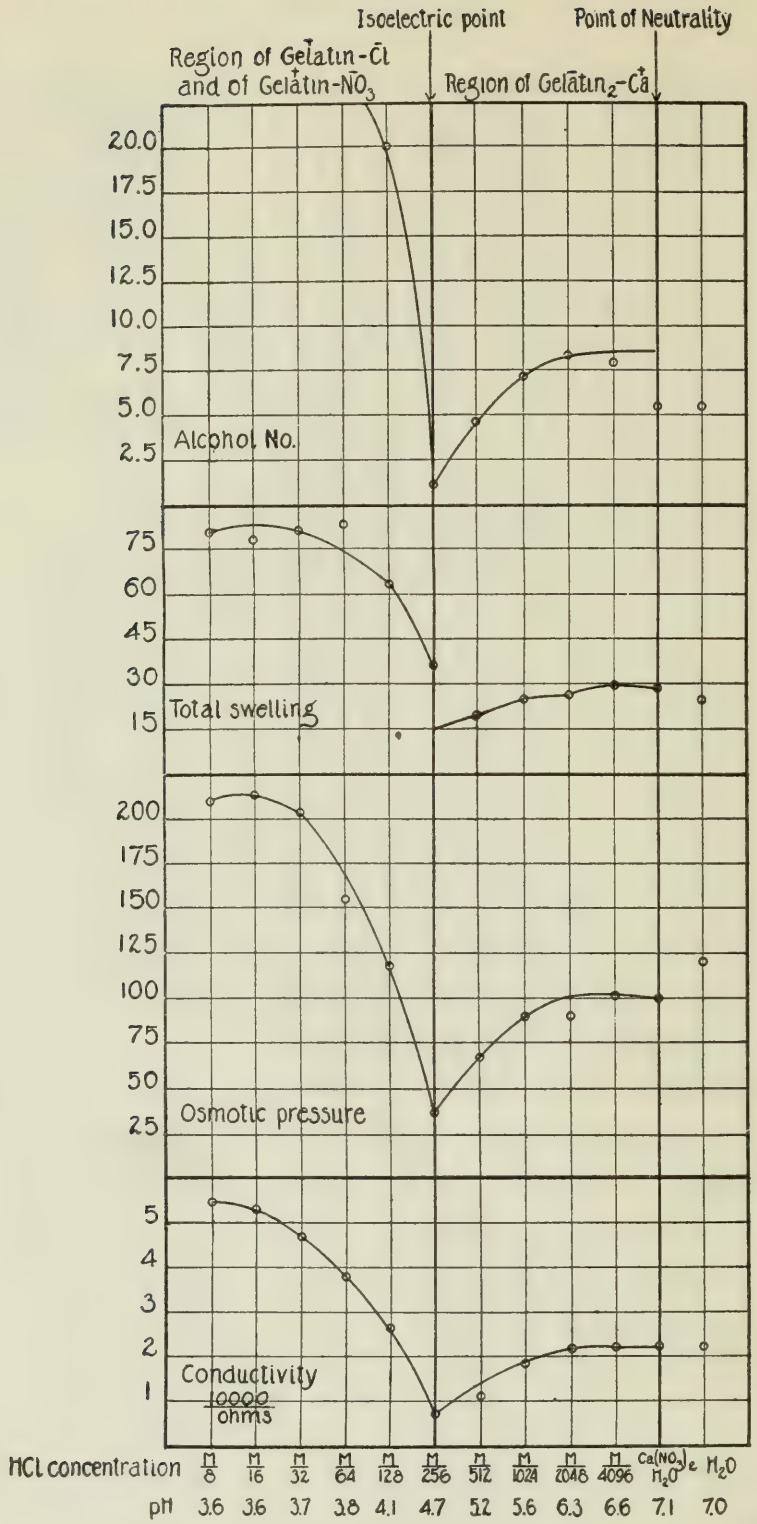


FIG. 3. $Ca(NO_3)_2$-HCl curves. Minima of all curves again at pH = 4.7 (isoelectric point). The branches of the curves for osmotic pressure, swelling, and alcohol number on the left, where gelatin exists as mixture of gelatin Cl and gelatin nitrate, higher than the branches on right of the isoelectric point, where gelatin exists as calcium gelatinate.

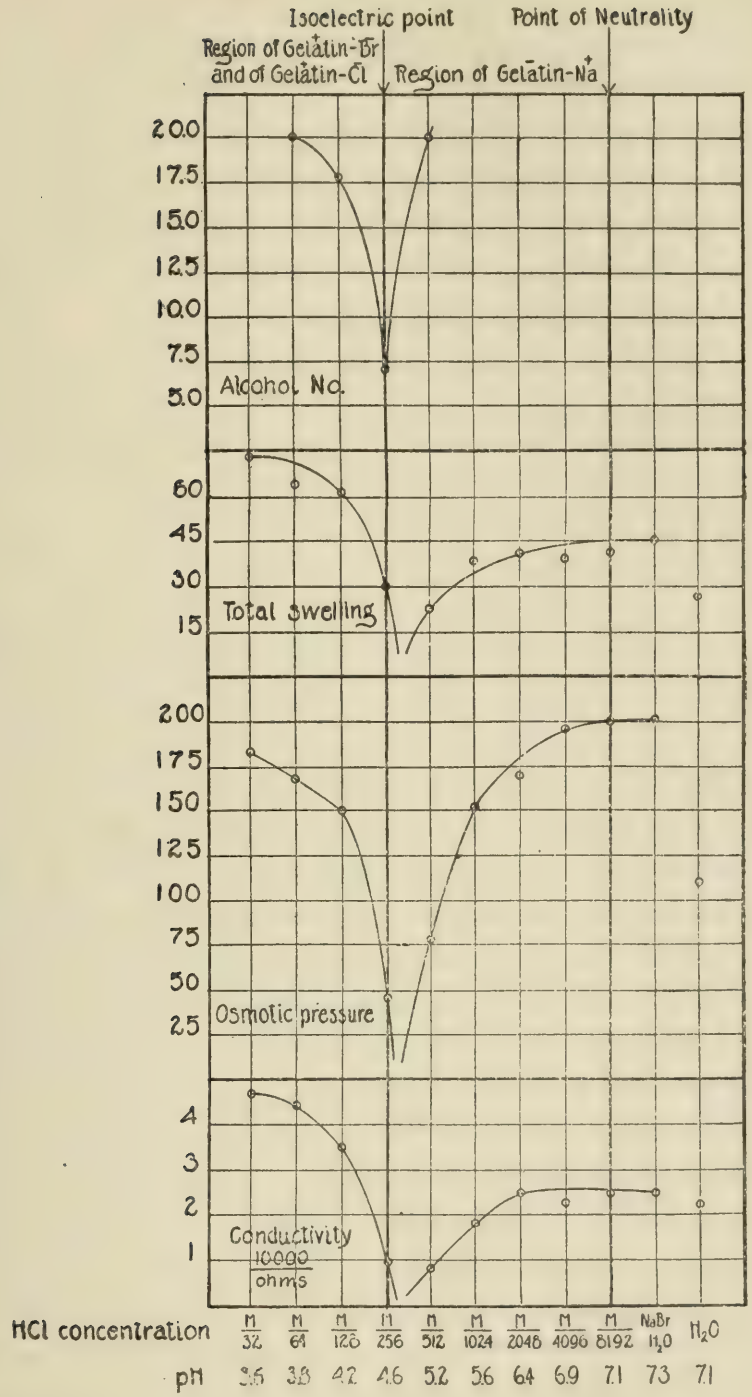


FIG. 4. NaBr-HCl curves. Minima again at pH = 4.7 (isoelectric point). Curves for osmotic pressure, swelling, and alcohol number almost symmetrical in regard to isoelectric point. On left side gelatin chloride and bromide, on right side sodium gelatinate.

then freed again from the salt by perfusion with water.[9] In this case the same results were obtained as those stated in this paper; namely: *When pH of the gelatin solutions is >4.7, the gelatin can exist in an ionized condition only as an anion capable of combining with cations only to form metal gelatinates, while on the more acid side of the isoelectric point (pH <4.7) gelatin can exist in an ionized form only as a cation capable of combining only with the anion of the neutral salt.* This law holds probably for all amphoteric electrolytes. The question then arises: How does an amphoteric electrolyte react with neutral salts at its isoelectric point? Before we answer this question we will point out an important fact shown by our curves, though this has no direct bearing on the subject under discussion.

III.

A comparison of the curves for the conductivities with those for the other properties of gelatin in Figs. 1 to 4 shows that the curves for conductivity are practically identical in all four figures as are also the values for pH; while the curves for osmotic pressure, etc., differ according to the valency of the ion combining with the gelatin. Since the conductivity curves are the direct expression of the degree of electrolytic dissociation, we can now say that the difference in the effect of univalent and bivalent ions demonstrated in the writer's previous publications is not merely a difference of the degree of ionization of the gelatin but is due to another effect upon the gelatin molecule. The important work by Langmuir and by Harkins on surface tension has suggested if not proved the idea of polarized molecules and of different degrees of solubility for different parts of the same organic molecule. It seems possible from their work that the solubility is confined to certain groups of the gelatin molecule or the protein molecule in general. It is quite possible that this will ultimately explain why the entrance of a univalent anion or cation into a gelatin molecule increases its solubility in water (or its attracting power for water molecules) and with this its osmotic pressure, alcohol number, viscosity, etc., while the entrance of a bivalent ion (com-

[9] In order not to increase the bulk of this paper we may be pardoned for omitting the details of these experiments.

bining possibly with two or more molecules of gelatin) will not cause or may even prevent such an increase, without altering the conductivity of the gelatin solution. The physical qualities of a gelatin solution depend, therefore, aside from the degree of its electrolytic dissociation and its hydrogen ion concentration upon a constitutional factor varying with the valency of the ion with which the gelatin is in combination. We intend to return to this question in a later publication.

IV.

The writer was curious to find out how gelatin reacts with neutral salts when brought to the isoelectric point. This was done by treating gelatin for 30 minutes with M/256 acetic acid or with M/256 or M/512 HCl, etc., and then perfusing the gelatin repeatedly with 25 cc. of H_2O in the way described. That the gelatin had reached the isoelectric point was ascertained by measurements of pH, of conductivity, osmotic pressure, etc. When gelatin on either side of the isoelectric point is treated with M/4 or M/8 NaCl or any other salt with univalent anion or cation, it shows an increase in osmotic pressure, swelling, alcohol number, after the salt is washed away; when, however, gelatin rendered isoelectric in the way described is treated with the same solution of NaCl, all its physical properties, conductivity, osmotic pressure, swelling, alcohol number, transparency, remain unaltered after the salt is washed away. It is immaterial whether we treat such gelatin with NaCl, Na_2SO_4, $CaCl_2$, or any other neutral salt; and it is also immaterial whether we use high or low concentrations of these salts; the physical qualities of the gelatin remain unaltered. Numerous experiments were made in which the nature of the acid, the concentrations, the number of washings, etc., used to reach the isoelectric point varied, but all yielded the same result, which means that *at the isoelectric point gelatin is chemically inert*, incapable of reacting with neutral salts. When, however, the pH of the gelatin deviates only slightly from that of the isoelectric point by becoming either $>$ or <4.7, the neutral salts react with gelatin in the way described in this and previous papers.

This is exactly what our theory demands, according to which gelatin can dissociate electrolytically only as an anion when pH is $>$ 4.7, as

a cation when pH is <4.7. Hence it can dissociate as neither when pH = 4.7. Not being able to dissociate electrolytically, it cannot react chemically with the ions of a neutral salt, as was found to be the case.

This inability to dissociate electrolytically and hence to react with neutral salts at the isoelectric point should be a common property of all amphoteric electrolytes and this seems to be the case. While this note goes to press a paper has appeared by Van Slyke and Baker,[10] containing the following sentence. "At the isoelectric point casein combines with neither acid nor base;" *i.e.*, with neither cation nor anion.

V.

The facts shown thus far support the idea that amphoteric electrolytes dissociate in a different way on the two sides of the isoelectric point; on the less acid side of that point they can ionize only as anions capable of combining with the cations of neutral salts, but not with their anions; on the acid side of the isoelectric point they can ionize only as cations capable of combining with the anion of a neutral salt, but not with its cation. The question then arises: What will happen when we bring a salt like sodium gelatinate in which gelatin is an anion on the acid side of the isoelectric point? Our theory demands that sodium gelatinate produced by treating gelatin with NaOH should not continue to exist when pH<4.7, and that in this case gelatin should behave as if it had not been treated with NaOH. This is what actually happens. The writer made this experiment some time before he had arrived at the theory published in this paper. He had undertaken the experiment in the expectation that gelatin first treated with alkali and then with acid should show a superposition of the effects of both treatments. 1 gm. of finely powdered gelatin was put for 30 minutes at 20°C. into each of a series of beakers all containing M/32 NaOH. After this, each powder was put on a filter, the alkali was allowed to drain off, and each filter was perfused four times with 25 cc. of H_2O to remove the last traces of NaOH held in the capillary spaces (except the NaOH formed by the hydrolytic dissociation of the sodium gelatinate). Then each filter was perfused three

[10] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 147.

times with 25 cc. of a HCl solution which varied for the different filters from M/8 to M/8192, and after this each filter was perfused four times with H_2O to remove the HCl not bound by the gelatin.

The result is given in the curves of Fig. 5 for the conductivity, osmotic pressure, swelling, and alcohol number. The curves show a marked minimum at about pH = 4.8; *i.e.*, the isoelectric point. The appearance of this low minimum of the values for conductivity, osmotic pressure, swelling, etc., characteristic of the isoelectric point, was at first a surprise and a puzzle to the writer. On the right side the curve of osmotic pressure rises to a value of 125 to 150 mm. which corresponds to the value for gelatin treated with M/32 NaOH and washed ten times with H_2O (without any subsequent treatment with HCl). On the acid side of the isoelectric point the curve of osmotic pressure rises to that point which corresponds to the effect the same concentration of HCl has on gelatin not previously treated with alkali. Hence the result of the acid treatment is on the left, more acid side from the isoelectric point, exactly the same as when we use gelatin not previously treated with M/32 NaOH and apply to it the same treatment with HCl as in this experiment; while on the right, less acid side of the isoelectric point, it is the same as if the gelatin had been treated with NaOH of increasing concentrations.

This permits of only one explanation, which in the light of the experiments published in the preceding parts of this paper is as follows. Sodium gelatinate, or perhaps more correctly a negative gelatin ion, cannot continue to exist except at hydrogen ion concentrations lower than that defining the isoelectric point. This means that in the acid solutions all or most of the sodium gelatinate must have given off its sodium ions and must have been retransformed into the weak gelatin acid; or some other constitutional (tautomeric?) change must have occurred in the gelatin molecule on the acid side of the isoelectric point. On the alkali side of the isoelectric point the sodium gelatinate could and did continue to exist.

This experiment was repeated with different concentrations of acids and alkalies, all confirming our conclusions. The experiment was also reversed, *i.e.* the gelatin was first treated with acid and then with alkali, and again our theory was confirmed.

It is of interest to see our view confirmed also by another set of facts. It has long been known that proteins and probably ampho-

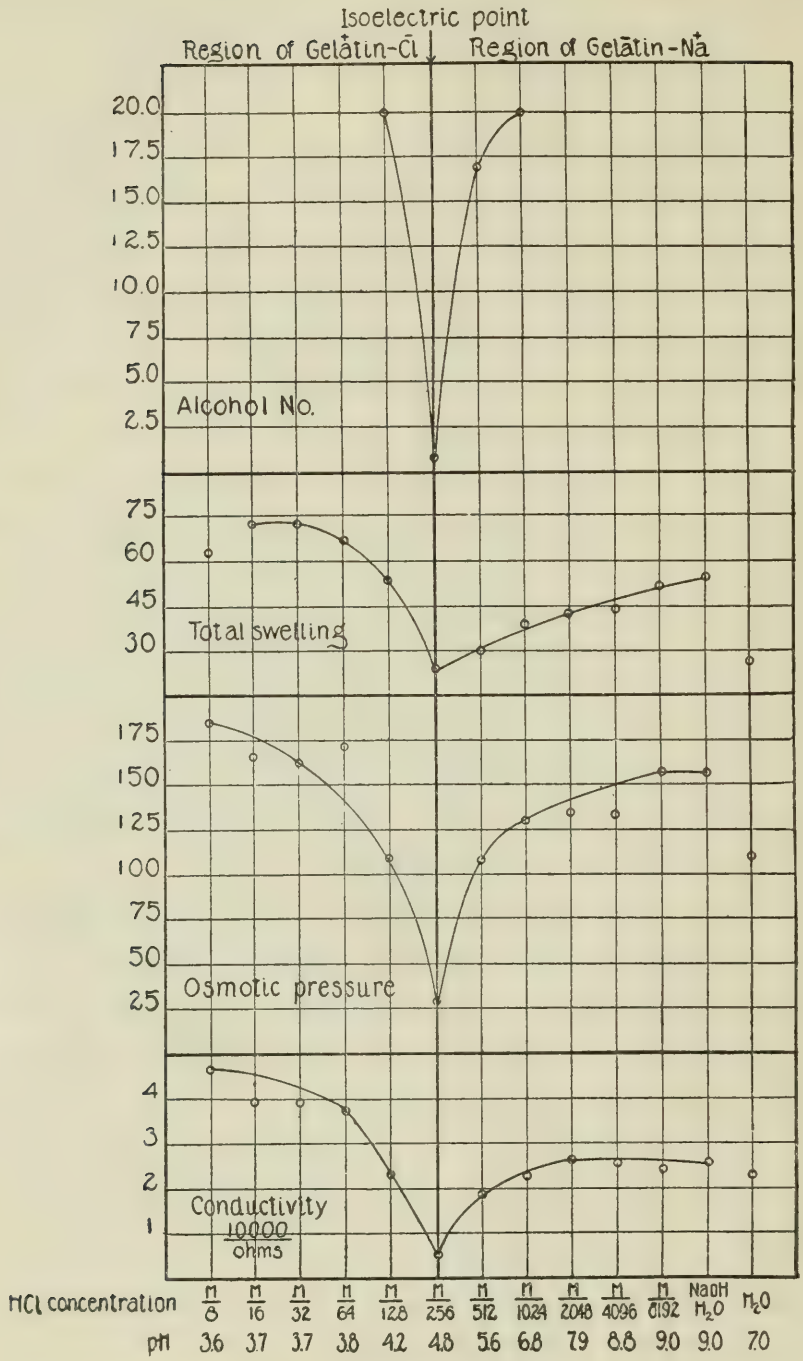


FIG. 5. M/32 NaOH followed by HCl treatment. Minima at pH = 4.8 (isoelectric point). Branches of curves on left side almost identical with those of Fig. 1 (where HCl alone was applied). On the left side of isoelectric point gelatin exists as gelatin chloride, on the right side as sodium gelatinate.

teric electrolytes in general stain with acid dyes in an acid but not in an alkali solution, and with basic dyes only in an alkaline solution. This agrees with our theory that an amphoteric electrolyte like gelatin can exist as an anion capable of combining with a basic dye (*e.g.* neutral red) only on the basic side of its isoelectric point, and that gelatin can exist as a cation capable of combining with acid dyes (*e.g.* acid fuchsin) only on the acid side of its isoelectric point. Our theory further demands that gelatin when stained with neutral red should give off its dye in solutions on the acid side of its isoelectric point, since on this side of the isoelectric point gelatin can no longer exist as an anion, while gelatin stained with acid fuchsin should give off its dye in solutions with a pH >4.7, since here the gelatin can no longer exist as a cation. All this turns out as our theory demands. The writer intends to make further experiments on this problem, but the facts thus far observed support our theory that when dissociating electrolytically an amphoteric electrolyte is stable as an anion only on the basic side of its isoelectric point, and as cation on the acid side of its isoelectric point. Of course, in a non-ionized condition gelatin may exist at any pH.

Another apparent support of our theory lies in the old observation made by Hamburger[11] on the absorption of Cl and SO_4 by the red blood corpuscles from serum when the CO_2 of the blood rises. In this case it is probably simply a question of raising the pH of the blood above that of the isoelectric point for hemoglobin, which is near the point of neutrality. The whole problem of the retention and excretion of electrolytes by the body may become intelligible from the fact proved in this paper that proteins or amphoteric electrolytes in general can exist as cations only (and have a tendency to become cations) on the more acid side of the isoelectric point, while they can exist as anions only (and have a tendency to become anions) on the less acid side of their isoelectric point.

[11] Hamburger, H. J., and van Lier, G. A., *Arch. Physiol.*, 1902, 492. Hamburger, *Biochem. Z.*, 1918, lxxxvi, 309. See also, Hasselbalch, K. A., and Warburg, E. J., *ibid.*, 1918, lxxxvi, 410.

SUMMARY.

1. It has been shown in this paper that while non-ionized gelatin may exist in gelatin solutions on both sides of the isoelectric point (which lies for gelatin at a hydrogen ion concentration of $C_H = 2.10^{-5}$ or pH = 4.7), gelatin, when it ionizes, can only exist as an anion on the less acid side of its isoelectric point (pH > 4.7), as a cation only on the more acid side of its isoelectric point (pH < 4.7). At the isoelectric point gelatin can dissociate practically neither as anion nor as cation.

2. When gelatin has been transformed into sodium gelatinate by treating it for some time with M/32 NaOH, and when it is subsequently treated with HCl, the gelatin shows on the more acid side of the isoelectric point effects of the acid treatment only; while the effects of the alkali treatment disappear completely, showing that the negative gelatin ions formed by the previous treatment with alkali can no longer exist in a solution with a pH < 4.7. When gelatin is first treated with acid and afterwards with alkali on the alkaline side of the isoelectric point only the effects of the alkali treatment are noticeable.

3. On the acid side of the isoelectric point amphoteric electrolytes can only combine with the anions of neutral salts, on the less acid side of their isoelectric point only with cations; and at the isoelectric point neither with the anion nor cation of a neutral salt. This harmonizes with the statement made in the first paragraph, and the experimental results on the effect of neutral salts on gelatin published in the writer's previous papers.

4. The reason for this influence of the hydrogen ion concentration on the stability of the two forms of ionization possible for an amphoteric electrolyte is at present unknown. We might think of the possibility of changes in the configuration or constitution of the gelatin molecule whereby ionized gelatin can exist only as an anion on the alkaline side and as a cation on the acid side of its isoelectric point.

5. The literature of colloid chemistry contains numerous statements which if true would mean that the anions of neutral salts act on gelatin on the alkaline side of the isoelectric point, *e.g.* the alleged effect of the Hofmeister series of anions on the swelling and osmotic pressure of common gelatin in neutral solutions, and the statement

that both ions of a neutral salt influence a protein simultaneously. The writer has shown in previous publications that these statements are contrary to fact and based on erroneous methods of work. Our present paper shows that these claims of colloid chemists are also theoretically impossible.

6. In addition to other physical properties the conductivity of gelatin previously treated with acids has been investigated and plotted, and it was found that this conductivity is a minimum in the region of the isoelectric point, thus confirming the conclusion that gelatin can apparently not exist in ionized condition at that point. The conductivity rises on either side of the isoelectric point, but not symmetrically for reasons given in the paper. It is shown that the curves for osmotic pressure, viscosity, swelling, and alcohol number run parallel to the curve of the conductivity of gelatin when the gelatin has been treated with acid, supporting the view that these physical properties are in this case mainly or exclusively a function of the degree of ionization of the gelatin or gelatin salt formed. It is pointed out, however, that certain constitutional factors, *e.g.* the valency of the ion in combination with the gelatin, may alter the physical properties of the gelatin (osmotic pressure, etc.) without apparently altering its conductivity. This point is still under investigation and will be further discussed in a following publication.

7. It is shown that the isoelectric point of an amphoteric electrolyte is not only a point where the physical properties of an ampholyte experience a sharp drop and become a minimum, but that it is also a turning point for the mode of chemical reactions of the ampholyte. It may turn out that this chemical influence of the isoelectric point upon life phenomena overshadows its physical influence.

8. These experiments suggest that the theory of amphoteric colloids is in its general features identical with the theory of inorganic hydroxides (*e.g.* aluminum hydroxide), whose behavior is adequately understood on the basis of the laws of general chemistry.

Addendum.—Since this paper went to print the writer has been able to show by volumetric analysis that the view expressed in this paper is correct; namely, that on the alkaline side from the isoelectric point gelatin combines with the cation of a neutral salt,

while on the more acid side from the isoelectric point it combines with the anion of a neutral salt. The curve for the amount of cation or anion in combination with the gelatin runs parallel with the curves for osmotic pressure, swelling, etc. The new experiments will be discussed in the second number of this *Journal.*

[Reprinted from The Journal of General Physiology, September 20, 1918, Vol. i, No. 1, pp. 81–96.]

THE LAW CONTROLLING THE QUANTITY OF REGENERATION IN THE STEM OF BRYOPHYLLUM CALYCINUM.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 24, 1918.)

I.

It is well known that isolated pieces of plants and lower animals are able to replace the lost parts by a new growth which is called regeneration. The investigation of this phenomenon by merely qualitative methods of experimentation has led only to verbalisms. Thus it has been stated by Noll that the plant or animal possesses a "morphestesia;" *i.e.*, a consciousness of what its proper form should be and hence the regeneration of lost organs. Driesch applies a similar verbalism calling the morphestesia "entelechy." Weismann suggests that without the power of regeneration many species might have died out—hence regeneration. Others speak of regeneration as the effect of the "stimulus" of the wound. If we wish to substitute for these or similar expressions, which have led and can lead to no scientific result, a theory in the sense in which this word is used by the physicist, we must investigate the problem of regeneration by the methods of quantitative experimentation.

The writer has introduced such a method in the study of regeneration of the plant *Bryophyllum calycinum*, which is unusually favorable for work of this kind, and he has already reported some of the results obtained. The most significant fact was found in the regeneration of the leaf of this plant. When the leaf of *Bryophyllum* is detached from the plant, it will form roots and shoots in its notches. Each node of the stem has two leaves in opposite position—sister leaves —which under normal conditions have equal size. Since they have also the same age and the same history, they possess not only the same mass but contain also very probably chloroplasts in the same number and in the same degree of efficiency; so we can say such sister leaves

have approximately equal masses of *active* substance. The writer was able to show *that such sister leaves of equal weight produce equal masses of shoots in equal times and under equal conditions of illumination, temperature, and moisture.* He found, moreover, that if we reduce the mass of one set of sister leaves by cutting out pieces from the center of the leaves, while the other set remains intact, both sets produce shoots approximately in proportion to their masses, even if the number of shoots produced by the two sets differs widely. We, therefore, can say that *equal masses of sister leaves produce equal masses of shoots in equal times and under equal conditions, regardless of the number of shoots produced.*[1]

This law shows that the problem of regeneration is part of the problem of growth and that it falls under the law of chemical mass action.

Inasmuch as age and previous history influence the active mass of photosynthetic material of the leaf, it is obvious that the law of the production of equal masses of shoots by equal masses of leaves is fulfilled more accurately by comparing sister leaves than it would be by comparing leaves with a different history; *i.e.*, leaves which differ in the mass of photosynthetic material in the unit mass of leaf. In such cases we should have to use statistical methods; *i.e.*, we should be compelled to use much larger numbers of leaves in order to eliminate the influence of variation in the relative mass of photosynthetic and other material in the leaf which is required for the regeneration (growth) of the shoots.

II.

Regeneration in an isolated piece of stem is much more general than regeneration in an isolated leaf, and the question arises whether a similar mass law, as that found for regeneration in a leaf, controls the quantity of regeneration in a stem.

In the axil of each leaf of the stem of *Bryophyllum* is found a dormant bud capable of growing into a shoot (3, Fig. 1). Each node contains two such dormant buds on opposite sides, one in the axil of each leaf. In successive nodes the lines connecting the two leaves

[1] Loeb, J., *Science*, 1917, xlv, 436; *Bot. Gaz.*, 1918, lxv, 150; *Ann. Inst. Pasteur*, 1918, xxxii, 1.

and their axillary buds are at right angles to each other. If we, therefore, split a stem longitudinally into two halves (Fig. 1) in such a way that the plane of section is at right angles to the line connecting the two most apical buds (1, Fig. 1), the section cuts through the buds in the next node (2, Fig. 1), and injures them more or less, while it leaves the buds of the second next (3, Fig. 1) node intact, and so on. It happens for reasons which need not be discussed here that in such a stem as a rule only the most apical bud (1, Fig. 1) grows out.

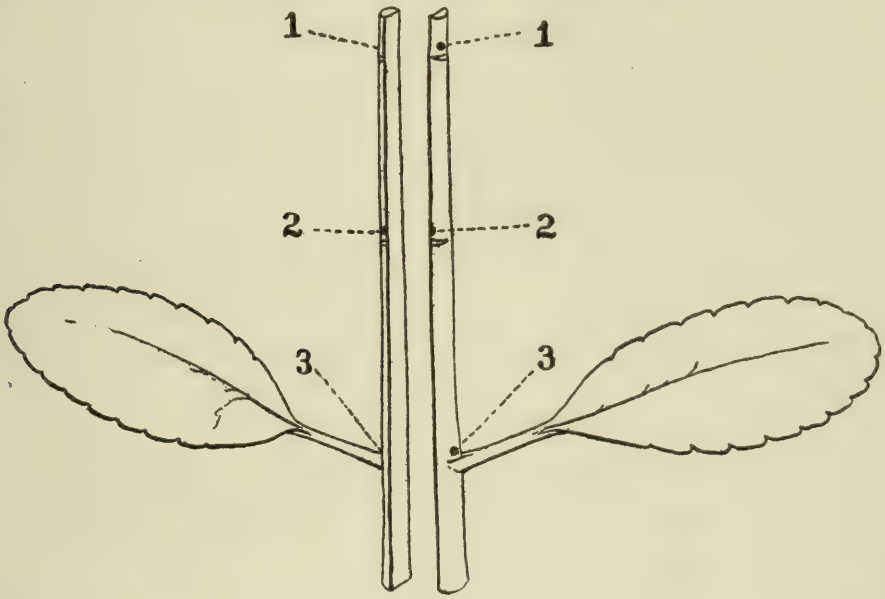


Fig. 1. Diagram showing the method of splitting the stem longitudinally in order to investigate the influence of mass of basal leaf upon shoot production. 1, 2, 3, designate the three pairs of dormant buds, two in each of the three nodes of the stem.

The following method was used for testing whether or not the quantity of regeneration in a piece of stem is controlled by the simple mass law which holds for the regeneration of shoots in the stem. From the stem of *Bryophyllum* were cut pieces containing three nodes (Fig. 1). All the leaves were removed except the two at the basal node. Then the stem was split lengthwise so that each half of the stem contained one basal leaf and one intact bud at the apex (1, Fig. 1), while the two buds (2, Fig. 1), in the middle node were generally injured in the operation. As stated, in most cases only the apical bud grows out in such a piece of stem. By leaving the leaf in one of

the half stems intact, while the sister leaf at the base of the other half stem is reduced in size, it is possible to find out whether the mass of the shoots regenerated at the apex of each half stem bears any relation to the mass of the two leaves. Such experiments were made by us and as a rule six or more different stems were used in one experiment.[2]

Many experiments are lost for the reason that the leaf at the base of a stem wilts or perishes much more rapidly than a leaf entirely detached from a stem; the latter may last for months while the former will usually wilt after a few weeks. For this reason it is more difficult to obtain exact results in investigating the influence of the mass of the leaf on the mass of the shoots formed at the apex of a stem than in investigating this influence upon the mass of shoots regenerated by isolated leaves. But in watching the condition of the leaves and realizing that we can only utilize an experiment when all the leaves remain intact we are able to obtain reliable results. This source of error due to wilting and decay of the leaf attached to the base of a stem which restricts the duration of the experiments is greater when the thin summer leaves are used than when the more fleshy and more durable winter leaves are utilized.

We shall first give the numerical results of some experiments in which only the fresh weight of the leaves and of the shoots regenerated by the stem was ascertained (Table I).

In Experiment 3 of Table I, the leaves of both half stems were left intact; 1 gm. of leaf in one set caused the production of 213 mg. of shoots in the stem, while 1 gm. of leaf of the other set produced 240 mg. of shoots in the apex of the stem. This shows the degree of accuracy to be expected in these experiments.

In the three other experiments the masses of the two sets of sister leaves varied considerably. Thus in Experiment 1 the masses of the leaves were approximately in the ratio of 1 : 6 (2.8 : 19.0); the masses of shoots produced by the two sets of stems were also approximately in the ratio of 1 : 6, namely 0.44 : 2.8. Experiment 2 gave similarly good results. The ratio of the two masses of sister leaves was approximately 1 : 5, namely 3.5 : 18.5, and the masses of shoots produced by the stem were also approximately in the ratio of 1 : 5, namely 0.7 : 3.6.

[2] Loeb, *Proc. Nat. Acad. Sc.*, 1918, iv, 117.

In Experiment 4 the two masses of sister leaves were in the ratio of almost 1 : 5, namely 2.7 : 11.9, and the masses of shoots produced by the stem were also approximately in the ratio of 1 : 5, namely 0.53 : 2.7.

Our experiments have shown without exception that the greater the mass of a basal leaf the greater the mass of shoot regenerated by the apex of the stem in equal times and under equal conditions; and wherever we were certain that the leaves remained normal during the experiment it was also possible to show that the mass of shoots produced by the apex of the stem

TABLE I.

Influence of Mass of Leaf at Base of a Piece of Stem upon Mass of Shoot Regenerated at the Apex of the Piece of Stem. Apices of Leaves Dipping in Water, Stems Suspended in Moist Air (See Fig. 2).

No. of experiment.	Duration of experiment.		Fresh weight of leaves.	Fresh weight of 6 regenerated shoots on stem.	Regenerated shoots per gm. of leaf.
	days		*gm.*	*gm.*	*mg.*
1	37	6 whole leaves	19.030	2.808	146
		6 sister " reduced in size	2.853	0.443	152
2	34	6 whole leaves	18.490	3.586	194
		6 sister " reduced in size	3.503	0.668	186
3	26	6 whole leaves	7.128	1.511	213
		6 " sister leaves	8.142	1.963	240
4	33	6 whole leaves	11.878	2.728	229
		6 sister " reduced in size	2.740	0.530	200

varied approximately in direct proportion to the mass of the leaf attached to the base of the piece of stem.

Fig. 2 is a drawing of the appearance of a group of six pairs of half stems having a leaf at the base. The duration of the experiment was 21 days. The upper half stem with a whole leaf is always the sister piece of the half stem with a reduced leaf immediately below it. It is obvious that the stems with the reduced leaves have smaller shoots than those with whole leaves. The apices of the leaves were dipped in water, the stems were in moist air. Fig. 3 is a photographic

reproduction of the same experiment (with left and right sides reversed).

These experiments prove that *equal masses of leaves at the base of a piece of stem cause the production of approximately equal masses of shoots at the apex of the stem in equal times and under equal conditions.*

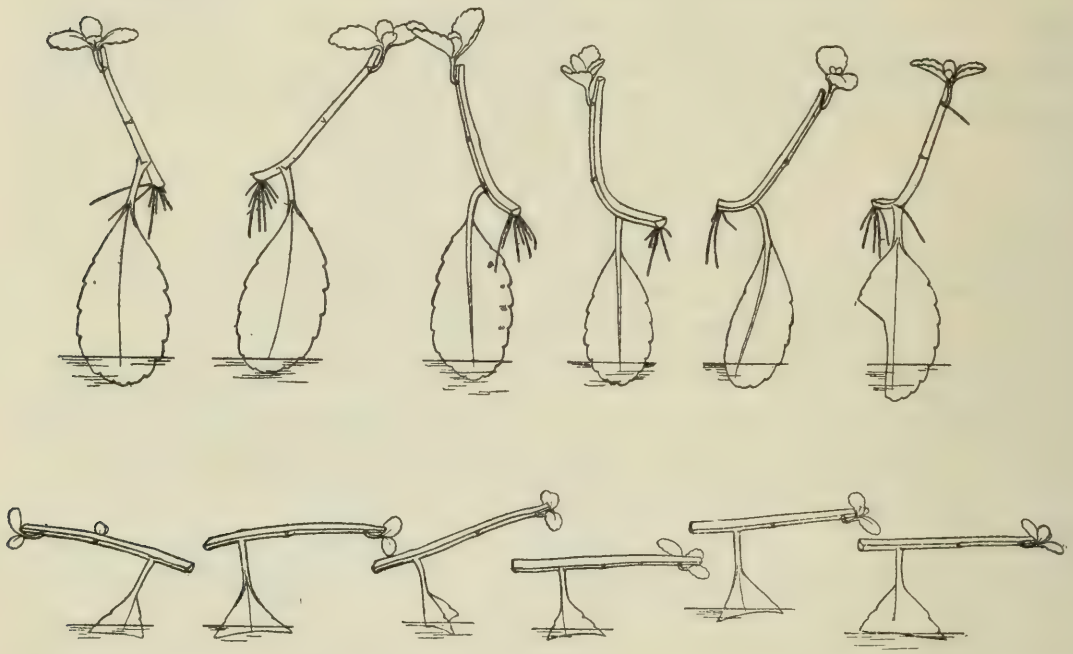


Fig. 2. Drawn after nature. The upper specimen with whole leaf and the one beneath it with reduced leaf are always the two halves of the same piece of stem split lengthwise in the way indicated in Fig. 1. The shoots at the apex of the stem with whole leaves (upper row) are considerably larger than the corresponding shoots with the sister leaf reduced in size (lower row). As a matter of fact, the masses of regenerated shoots were in proportion to the masses of the leaves attached to the base of the stems. The reader will also notice that the stems with whole leaves (upper row) have formed roots at their base, while those with reduced leaves have not yet formed any roots (though they did so later). It is also noticeable that the shoots with whole leaves show geotropic curvature, while those with reduced leaves are still straight.

Even where some of the leaves were wilted the mass of shoots produced at the apex was always greater when the mass of leaves was greater, but it was no longer possible to prove approximate proportionality though such proportionality in all probability existed.

In the experiments presented in Table II the stems as well as the leaves were suspended in moist air to eliminate the influence of ab-

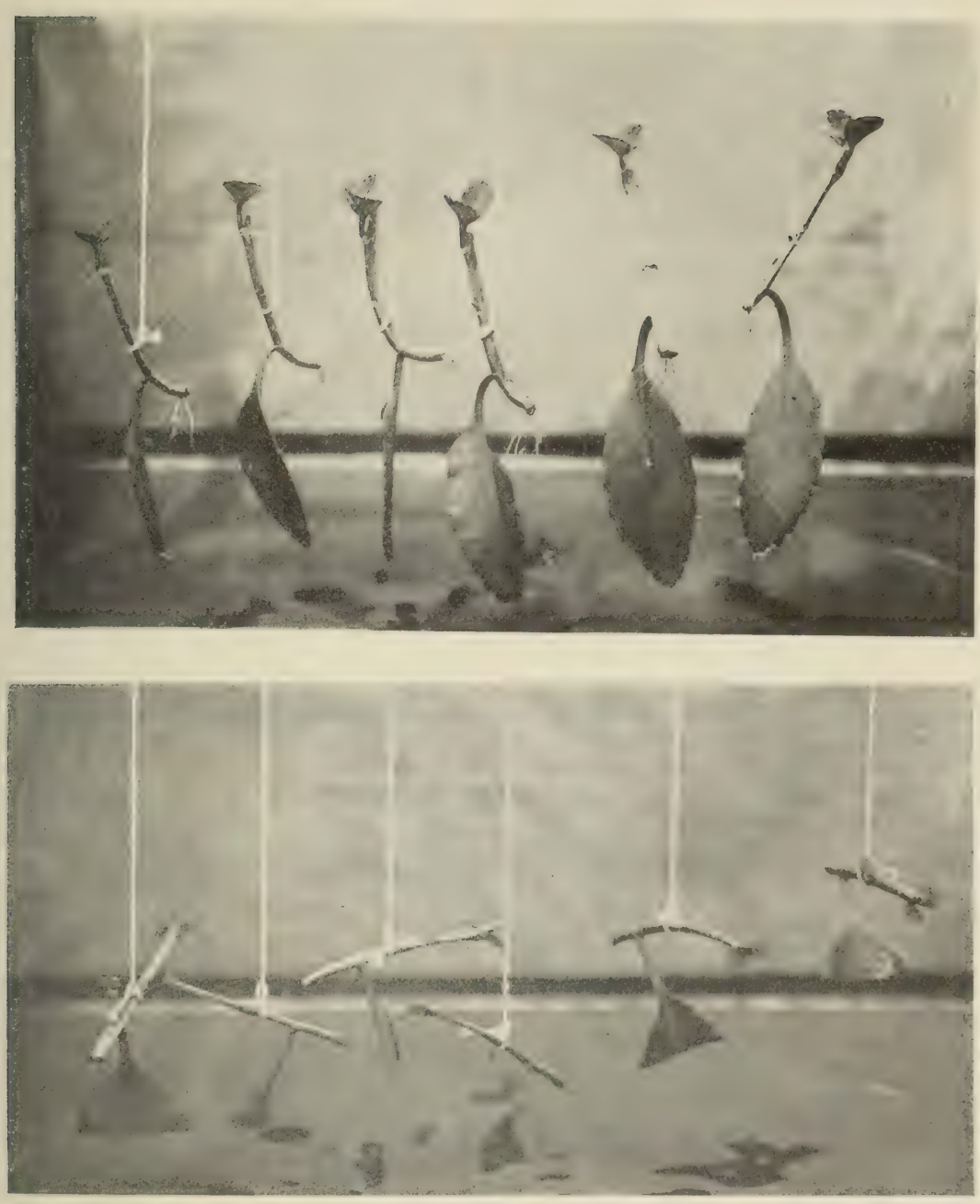


Fig. 3. Photograph showing the same experiment as in Fig. 2. (Left and right are reversed in Figs. 2 and 3.)

sorption of water by the leaf. The duration of these experiments had to be short on account of the more rapid wilting of the leaves connected with the stems.

In this case the disturbing factor of wilting is greater than when the apices of the leaves dip into water and hence the results with leaves suspended entirely in air show a smaller approximation to proportionality between mass of leaves and mass of shoots produced by the stem than that demonstrated in Table I, although the proportionality is not entirely obliterated.

TABLE II.

Stems and Leaves Suspended in Moist Air.

No. of experiment.	Duration of experiment.		Fresh weight of leaves.	Fresh weight of 6 regenerated shoots on stem.	Regenerated shoots per gm. of leaf.
	days		*gm.*	*gm.*	*mg.*
1	17	6 whole leaves	4.376	0.524	119
		6 " sister leaves	3.264	0.494	151
2	19	6 whole leaves	6.662	1.126	168
		6 sister " reduced in size	2.853	0.6885	237

III.

The mass of shoot produced in an isolated piece of stem entirely deprived of leaf is small compared with that produced when a leaf is attached to the base of the stem. This was demonstrated in the following experiment.

Six stems, each containing three nodes and one leaf at the base, were split longitudinally in the way described above. One half stem contained one leaf at the base, the other had no leaf. To insure an equal water supply to the stems they were put with their cut sides on moist filter paper (Fig. 4), the leaf at the base of the one set of stems being in moist air. The duration of the experiment was 24 days.

Fresh weight of shoots produced in 6 half stems *without leaves*, 0.120 gm. (dry weight, 0.0105 gm.).

Fresh weight of shoots produced in 6 half stems *with leaves*, 2.088 gm. (dry weight, 0.2015 gm.).

Weight of 6 leaves, 8.262 gm.

The dry weight of the 6 stems without leaves was 0.286 gm. and the fresh weight was 3.935 gm. The dry weight of the 6 half stems containing a leaf was 0.688 gm. and the fresh weight 6.203 gm. We have already shown in a previous paper that the mass of an isolated piece of stem increases when a leaf is attached to t.[3]

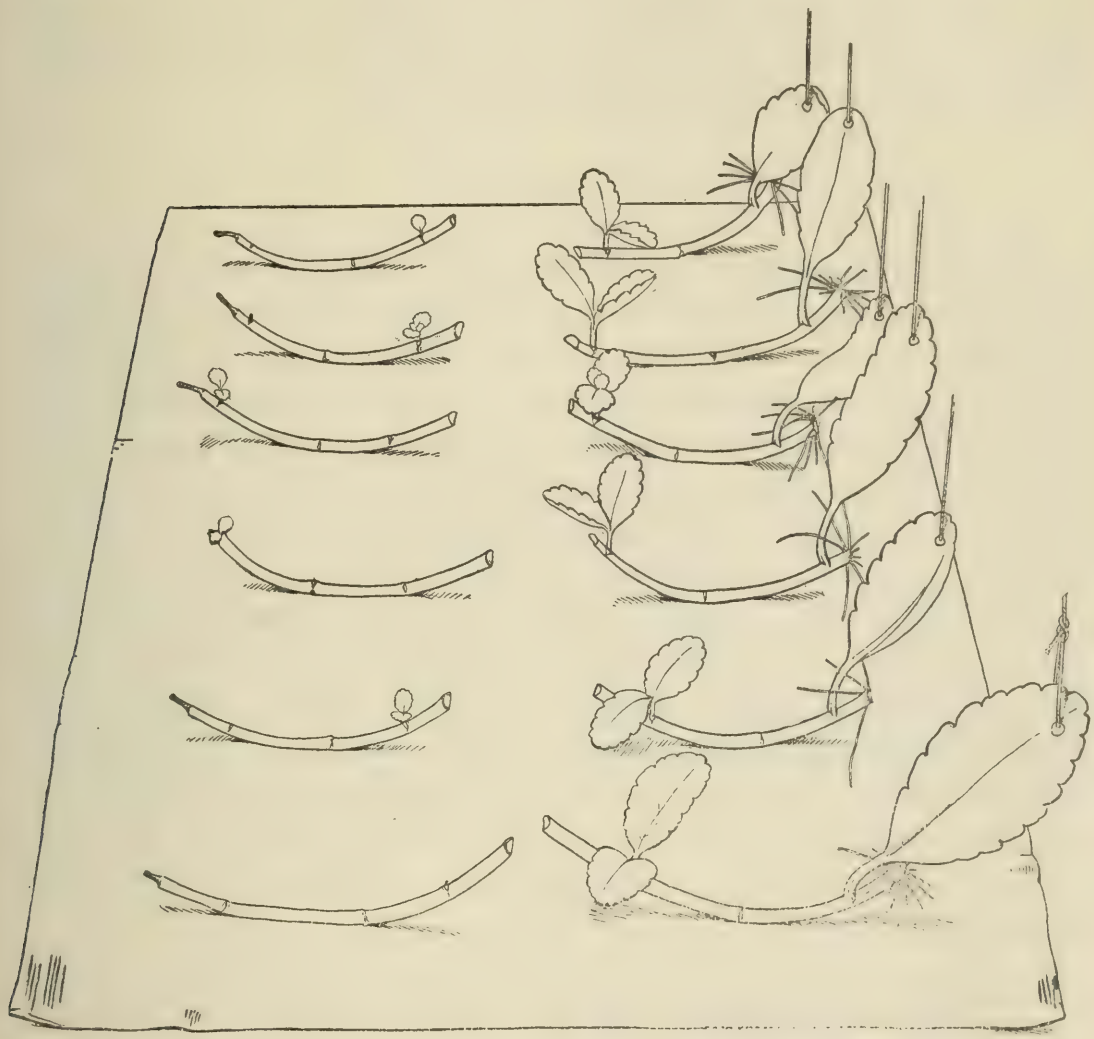


Fig. 4. Showing the enormous difference in mass of shoots regenerated by a half stem with leaf at base and the other half stem without leaf. The stems rested with their cut surface on moist filter paper.

Hence we can say that only about 5 per cent of the material of the regenerated shoots was contributed by the stem and about 95 per cent of the mass of the regenerated shoots of the stem was formed from

[3] Loeb, *Ann. Inst. Pasteur*, 1918, xxxii, 1.

material furnished by the leaf. This is not surprising since we know that the leaf is the organ where the material for new growth in the plant is manufactured. It is also to be expected that in pieces of stems of different mass but without leaves the mass of shoots regenerated will increase with the mass of the piece of stem. The writer has already published experiments indicating that this is true,[4] though he has not yet made quantitative determinations to find out whether the law of proportionality holds in this case also.

IV.

The law of proportionality between mass of the leaf attached to the base of a stem and mass of shoot produced apically from the leaf can be proved for other cases also. If we cut out a piece of stem with only one node containing two leaves such a piece possesses only two buds capable of developing into shoots; namely, one in each of the axils of the two leaves (Fig. 5). These axillary buds grow out more rarely and more slowly than the free buds at the apex of a piece of stem. Fourteen pieces of stem with one node and two leaves each were cut out from plants and each piece of stem was split longitudinally between the two leaves. One leaf remained always intact, the other leaf was reduced by cutting off part of the leaf (Fig. 5). Eight of these fourteen pieces of specimens formed axillary shoots. It seemed of interest to find out whether the mass of these shoots was approximately in proportion to the mass of the leaves. This was the case (Fig. 5). The duration of the experiment was 45 days. The apices of the leaves dipped into water.

a. Weight of 8 whole leaves, 10.968 gm. Weight of 8 shoots produced in their axil, 1.8025 gm. Mg. of shoots produced per gm. of leaf, 164.

b. Weight of 8 reduced sister leaves, 3.586 gm. Weight of 8 shoots produced in axil, 0.5895 gm. Mg. of shoots produced per gm. of leaf, 164.

The mass of axillary shoots produced by each set of sister leaves was, therefore, in direct proportion to the mass of the leaves.

[4] Loeb, *Bot. Gaz.*, 1915, lx, 249.

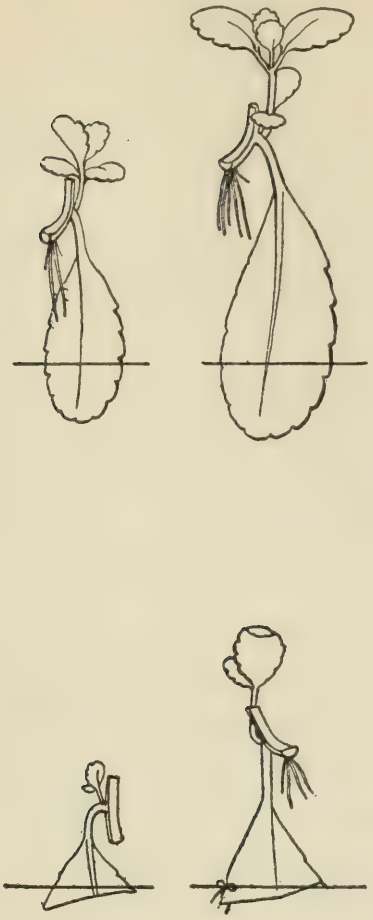


Fig. 5. Showing influence of mass of leaf on mass of shoot produced in the axil of each leaf. Each upper specimen with whole leaf and the specimen below it with reduced leaf are halves of the same stem.

V.

Since it might be argued that the leaves furnish only water for the growth of the shoots, it was necessary to find out whether the dry weight of the shoots regenerated by the stem varies also with the dry weight of the leaves attached to the base of the stem. The dry weight of the basal leaves and of the shoots regenerated by the stem was ascertained by drying these organs in an electric oven over night at 100°C. Some of the experiments mentioned in Tables I and II were used for this purpose and in Table III the experiments in which the dry weights were determined are indicated.

The leaves, therefore, do not only supply water for the regeneration of the shoots by the apex but dry matter as well. This conclusion is supported by the experiments to be discussed in the next chapter.

TABLE III.

No. of experiment.		Dry weight of leaves.	Dry weight of shoots.	Dry weight of shoots per gm. of dry weight of leaf.
		gm.	*gm.*	*mg.*
4, Table I.	6 whole leaves	0.440	0.2135	0.485
	6 sister " reduced in size	0.1055	0.0430	0.407
2, Table II.	6 whole leaves	0.3700	0.1002	0.271
	6 sister " reduced in size	0.1991	0.0632	0.317
1, Table II.	6 whole leaves	0.3144	0.0486	0.154
	6 " sister leaves	0.2585	0.0450	0.177

VI.

If it is true that the leaf furnishes the material to the stem from which the regenerating shoot grows, it should be possible to show that the basal leaf in connection with a regenerating stem has (after some time) less weight than the sister leaf which is separated entirely from the stem. This can indeed be demonstrated. A piece of stem is cut from a plant and all the leaves are removed except the two leaves at the basal node of the stem. One basal leaf is entirely separated from the stem to serve as a control; the other leaf remains in connection with the stem.

Six stems, about 6 cm. long, with several nodes and with a basal pair of leaves of approximately or practically the same size, were selected for the experiment. Such sister leaves of the same size have also practically the same mass as shown in previous experiments. Both the isolated leaves and the stems with one leaf attached were suspended in moist air, in the same aquarium under identical conditions of light, temperature, and moisture. After 16 days the fresh and dry weights of the two sets of leaves, namely of the detached leaves and of their sister leaves connected with the stem, were ascertained. It was found that the leaves connected with the stems weighed considerably less than the detached leaves, and the difference was far in excess of the natural variation in the weight of fresh sister leaves of equal size.

Experiment I. Leaves and Stems in Moist Air.

	Fresh weight. gm.	Dry weight. gm.
6 detached leaves, at end of experiment	8.242	0.826
6 sister leaves connected with stem, at end of experiment	6.476	0.578

Hence the leaves connected with the base of the stem had given off to the latter about one-third of their dry weight.

During this time the detached leaves formed roots and tiny shoots in some of their notches while nothing of this kind occurred in the leaves connected with the stems. Instead twelve shoots were formed at the apices of the six stems and the twelve shoots weighed fresh 0.528 gm. and dry, 0.068 gm. The inference is that the material for the latter came from the leaves, but apparently more material than this was given off by the leaves to the stems. We have indeed shown in a previous paper that aside from the material for shoot formation the leaf sends material into the stem which may be used for the growth of certain tissues in the stem, resulting in callus formation, geotropic curvature, and increased thickness of the stem.[3]

The experiment was repeated with this difference, that the apices of the leaves dipped into water, while the stem and the rest of the leaves were suspended in moist air. Both sets of leaves were in the same aquarium under equal conditions of temperature, light, and moisture, and both were of equal size and mass at the beginning of the experiment. The experiment lasted 18 days.

Experiment II. Apices of Leaves Dipping in Water; Stems Suspended in Moist Air.

	Fresh weight. gm.	Dry weight. gm.
6 detached leaves, at end of experiment	11.159	0.791
6 sister leaves attached to base of stem, at end of experiment	4.485	0.388

Hence the leaves gave off to the stem about 50 per cent of their dry weight.

This difference is only partially accounted for by shoot production in the stem, the total shoot production amounting to 1.166 gm. fresh and 0.111 gm. dry weight.

Many more experiments than these were made and in some the leaves were weighed both at the beginning and at the end of the

experiment.[5] They leave no doubt of the fact that the basal leaf gives off a considerable amount of its material to the stem.

We have, therefore, a right to conclude that a basal leaf gives off material to the stem, part of which is used for the formation of shoots in the stem.

Experiments were carried on in the dark and it was found that the mass of shoots regenerated under these conditions was small even when a large basal leaf was attached to the stem; and no proportionality between mass of leaf and of shoots regenerated on the stem could be found. This indicates that the products of assimilation in the leaf are part of the material used for the regeneration of the shoots on the stem.

Theoretical Remarks.

These experiments have shown that in the case of the regeneration of the leaf as well as of the stem the quantity of regeneration is determined by the mass of material sent out by the leaf and manufactured in the leaf; and possibly also to a small extent by material manufactured in the stem or circulating or stored in the stem at the time the stem was cut out from the plant.

The same simple mass law seems to hold for the quantity of roots regenerated at the basal end of the stem or basally from a leaf attached to the stem. It was very obvious in all our experiments that the mass of roots formed in a piece of stem in a given time increases with the mass of a leaf and the roots commence to grow out later when the leaf is smaller. The writer has, however, not yet made enough quantitative measurements to permit him to state that there is a strict proportionality between mass of leaf and mass of roots regenerated.

In animals the blood and lymph play the same rôle as does the sap in plants, and we may surmise that the quantity of sugar, amino-acids, salts, and of "accessory substances" in the body fluids determines the quantity of regeneration in animals. In an animal, regeneration may occur even when no food is taken up, and it is to be assumed that the tissues of a fasting organism constantly convert some of the material stored in the cells into sugar, amino-acids, and vitamines,

[5] We will omit these experiments here since they will be discussed in one of the following papers.

which diffuse into the blood and become available for the growth of the regenerating tissues; in other words, it is the constant hydrolysis going on in the organism which supplies the material for the growth and regeneration of fasting animals. It remains for future investigations to find out whether the ratio between mass of growth material circulating in the blood or lymph and the quantity of regeneration obeys also the simple mass law established for regeneration in *Bryophyllum*.

For those interested in the dynamics of these processes attention may be called to the fact that in our experiments the action of two masses of active material, m and m_1, in two sister leaves is compared. These masses are certain constituents of the two sister leaves, primarily the chlorophyll, and their ratio $\frac{m}{m_1}$ may be considered approximately constant throughout the duration of the experiment. Our experiments have furnished the proof that the ratio of the mass of shoot regeneration in two halves of a stem (each possessing a leaf at the base) is approximately proportional to $\frac{m}{m_1}$; or in other words, that the law controlling the quantity of shoot regeneration of the stem is a special case of the law of mass action.

SUMMARY.

1. A method is given which allows us to measure the influence of the mass of a leaf upon the quantity of shoots regenerated in an isolated piece of stem. This method consists in isolating a piece of stem with only two leaves left at the basal node and then splitting the stem lengthwise so that each half has one basal leaf. By leaving one leaf intact while the size of the sister leaf is reduced, the influence of the mass of the leaf upon the quantity of shoots regenerated by the stem can be measured.

2. This method has yielded the result that the mass of shoots regenerated at the apex of such a piece of stem increases under equal conditions and in equal time with the mass of the leaf, and is approximately proportional to the mass of the leaf.

3. Such an influence of the mass of the leaf upon the mass of shoots produced by the stem is only intelligible on the assumption that the

growth of the regenerating shoot occurs at the expense of material furnished by the basal leaf.

4. This assumption is supported by two facts: first, that in the dark this influence of the leaf disappears more or less completely; and, second, that a leaf attached to the base of a regenerating stem after some time weighs markedly less than does a sister leaf completely detached from the stem, but otherwise under equal conditions.

5. This latter fact that a leaf when attached to the base of an excised piece of stem wilts more rapidly than when completely isolated is the reason that the proportionality between mass of a basal leaf and mass of shoot regenerated at the apex of an isolated piece of stem cannot always be demonstrated with the same degree of accuracy as the proportionality between the mass of completely isolated leaves and the mass of shoots they produce.

6. The material furnished by the leaf to the stem is not restricted to water but includes also the solutes, since not only the fresh weight but also the dry weight of the shoot regenerated by a piece of stem increases with the mass of the leaf attached to the base of the stem; and since not only the water contents but the dry weight of a leaf attached to the base of an excised piece of stem diminish when compared with the dry weight of a completely detached sister leaf.

7. The mass of shoots produced by an isolated piece of stem without leaf is small and almost negligible compared with the mass of shoots produced by the same piece of stem when a leaf of sufficient mass is attached to the base of the stem.

[Reprinted from The Journal of General Physiology, November 20, 1918, Vol. i, No. 2, pp. 237–254.]

AMPHOTERIC COLLOIDS.

II. Volumetric Analysis of Ion-Protein Compounds; the Significance of the Isoelectric Point for the Purification of Amphoteric Colloids.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, August 28, 1918.)

INTRODUCTION.

In order to understand the influence of the hydrogen ion concentration upon the chemical and physical behavior of proteins we may well keep in mind the rôle of the hydrogenion concentration upon the reactions of an amphoteric metal hydroxide, like aluminium or chromic hydroxide, whose chemical constitution is better known than that of the proteins.

"Aluminium hydroxide dissolves in acids. From its solution in hydrochloric acid, an aluminium salt, aluminium chloride $AlCl_3$, $6H_2O$, is obtained. It also combines with strong bases, dissolving for instance in a solution of sodium hydroxide and forming an aluminate, $NaAlO_2$. . . . According to the best knowledge we have on the subject, the molecule of aluminium hydroxide has the following structure or arrangement of its atoms $Al(-O-H)_3$. It is readily seen that the cleavage of the molecules may produce, either aluminium and hydroxide ions, characteristic ions of a base, or aluminate and hydrogen ions, characteristic ions of an acid:

$$Al^{+++} + 3\,\overline{O}H \rightleftharpoons Al(-O-H)_3 \rightleftharpoons Al\overline{O}_2 + H^+ + H_2O."[1]$$

It is obvious that between the action of acids and of bases producing these two types of electrolytic dissociation there must be one hydrogen ion concentration in which aluminium hydroxide is practically neither able to form Al^{+++} nor $Al\overline{O}_2$ ions, and this would be the isoelectric point.

[1] Stieglitz, J., The elements of qualitative chemical analysis, New York, 1911, 171.

We may apply the experience gained from aluminium hydroxide to proteins which are also amphoteric electrolytes and for some of which the exact location of the isoelectric point is known. For gelatin it lies at pH = 4.7. In a previous paper[2] it was shown that when pH > 4.7, gelatin is only able to dissociate as gelatin (capable of combining with metals to form metal gelatinates, *e.g.* sodium gelatinate); when pH < 4.7, gelatin is only capable of dissociating as gelatin (capable of combining with anions to form, *e.g.*, gelatin chloride). At the isoelectric point it is practically not dissociated at all. There is this difference between amphoteric electrolytes of the type of proteins and that of metal hydroxides, namely that in the case of proteins the OH ions and the H ions are attached to different groups in the molecule.

We also have shown that a metal gelatinate must give off its metal ion when the hydrogen ion concentration is raised beyond that of the isoelectric point; and that a gelatin-anion compound must give off its anion when the hydrogen ion concentration is lowered below that of the isoelectric point. At the isoelectric point gelatin cannot be in combination with either anion or cation and such gelatin free from *ionogenic* impurities we designate as pure gelatin. It would thus appear that we can free proteins and amphoteric colloids in general from *ionogenic* impurities by bringing the ampholyte to its isoelectric point. Such impurities, however, as form part of the complex gelatin ion can, of course, not be removed by this method. In this and some following papers we intend to furnish additional proof for the correctness of these ideas. This can be done by making the gelatin combine with such ions as Ag or Br or CNS, etc., whose quantity can be easily determined volumetrically.

The Combination of Cation with Gelatin.

In all experiments in which the chemical or physical influence of an electrolyte upon an amphoteric colloid is to be investigated it is necessary to remove the excess of electrolyte after it has had time to act on the colloid; and the writer has shown that the neglect of this precaution has caused a good deal of the confusion which prevails in the

[2] Loeb, J., *J. Gen. Physiol.*, 1918, i, 39.

literature of colloid chemistry. By using gelatin in the condition of a fine powder we are able to wash away the excess of electrolyte after it has had time to act on the gelatin.

When we put 1 gm. of powdered gelatin for 30 minutes at 20 or 15° into 100 cc. of 3 M/1024 HCl or HNO_3, and then wash the gelatin on the filter to remove the excess of acid, we obtain a gelatin with pH of about 4.7; *i.e.*, of the isoelectric point. When we use a higher concentration than 3 M/1024 HCl, we get a pH $<$ 4.7, and a gelatin which is on the acid side of the isoelectric point and which is able to act only as cation; when we use an acid less concentrated than 3 M/1024, we get after the excess of acid is washed away a gelatin whose pH $>$ 4.7 and which being on the alkaline side of its isoelectric point can act only as an anion and can combine only with a cation.

In order to get clear results it is well to work with a series of different concentrations of an acid, *e.g.* M/4, M/8, M/16, etc., to M/8192 HCl or HNO_3, so that we obtain series of gelatin solutions with different pH on either side of the isoelectric point. After the excess of acid is washed away by several perfusions with H_2O, the gelatin is perfused three times with 25 cc. of the same solution of a salt, *e.g.* M/8 NaBr or M/8 NaCNS or M/16 $AgNO_3$, to bring about a reaction between salt and gelatin. It is necessary to stir up the gelatin on the filter while the salt solution is poured on to bring about as complete a contact between the powdered particles of gelatin and salt as possible. After the salt solution has drained off, the excess of salt is washed away by six perfusions with 25 cc. of H_2O each. The gelatin is then melted and made into a 1 per cent solution whose pH is determined and which is then analyzed for Br or silver, as the case may be. We shall describe some of the results obtained by this method.

1 gm. of finely powdered gelatin is put for 30 minutes at 15°C. into each of a series of beakers containing 100 cc. of HNO_3 varying from M/8 to M/8192; and into one beaker containing 100 cc. of distilled water serving as a control. The gelatin is then put on a filter, washed, and is perfused three times with 25 cc. of M/16 $AgNO_3$ in the way described, and subsequently six times with 25 cc. of H_2O to wash away the excess of $AgNO_3$. The swelling is measured while the gelatin is still in the cylindrical funnel, and the gelatin is melted and made into a 1 per cent solution. This part of the experiment is of course carried

out in a dark room. Then the pH is determined, and 25 cc. of the solution are used to determine the quantity of silver in combination with the gelatin, according to Volhard's method.

Fig. 1 contains two curves the abscissæ of which are the logarithms of the concentrations of HNO_3 with which the gelatin was originally

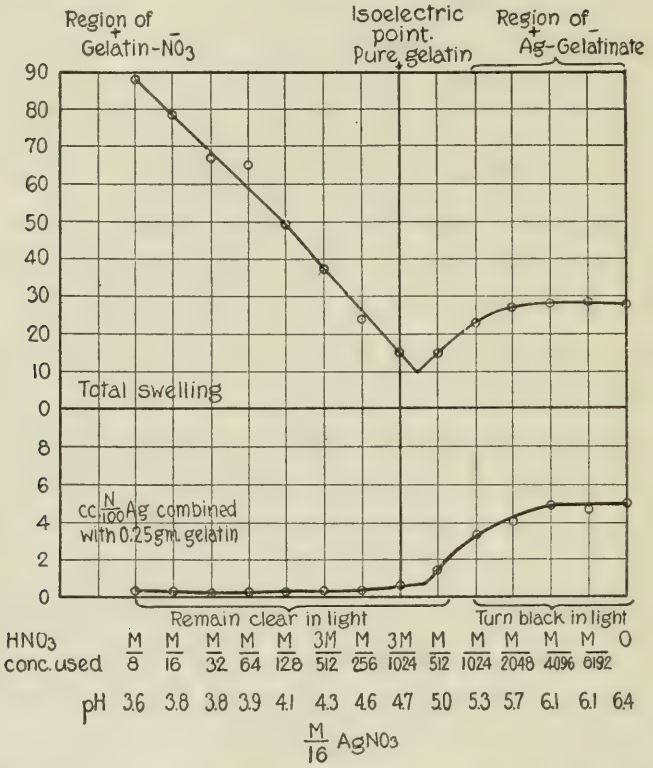


FIG. 1. Gelatin treated with different concentrations of HNO_3, from M/8 to M/8192, washed, and then treated with the same concentration of $AgNO_3$ (M/16), and then washed again. Abscissæ show concentrations of acid used. The final pH of the gelatin solution is found under the figure for the concentration of acid used.

The ordinates of the lower curve give the values for the silver found in combination with the gelatin. The curve shows that at the isoelectric point (pH = 4.7) and on the acid side of the isoelectric point, the gelatin was practically free from silver. On the more alkaline side the amount of silver found in combination with the gelatin increased with the pH. This proves that gelatin can combine with a cation only on the alkaline side from the isoelectric point, and this is corroborated by the fact that on the alkaline side from the isoelectric point only was the gelatin darkened by light. The ordinates of the upper curve are the values for the swelling of the same gelatin. On the alkaline side from the isoelectric point, where the gelatin had combined with silver, the curve for swelling runs parallel to the curve for silver gelatinate formed. It was, therefore, the relative mass of silver gelatinate formed which determined the physical properties of gelatin.

treated. Under each concentration is put the pH found for the gelatin solution at the end of the experiment. The ordinates of the lower curve are the numbers for the cc. of 0.01 N silver in combination with 0.25 gm. of gelatin. The curve shows that at the isoelectric point (pH = 4.7) the gelatin is free from ionic silver and that the same is true for all the gelatin on the acid side from the isoelectric point (pH 3.6 to 4.7). On the alkaline side from the isoelectric point the

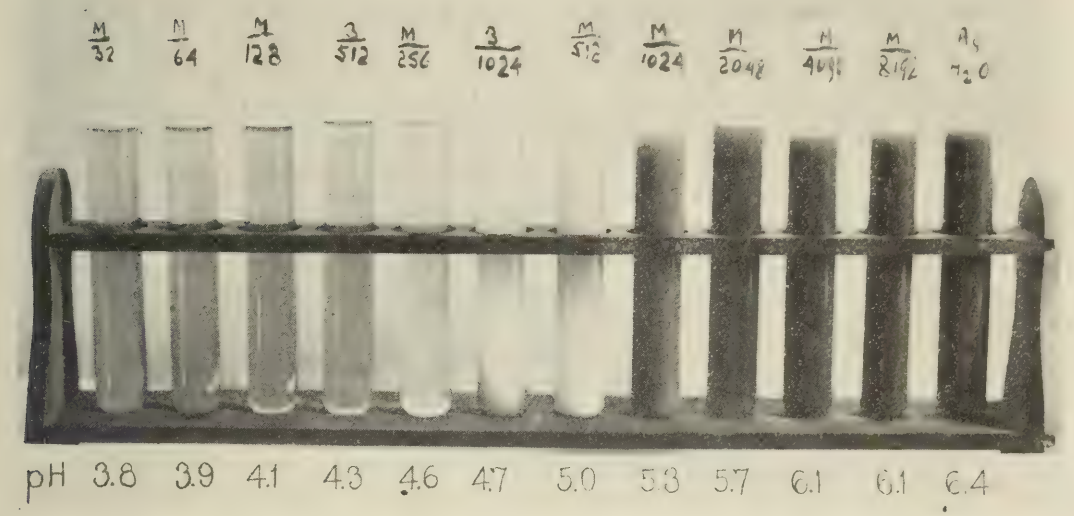


FIG. 2. Photograph of the gelatin solutions whose curves are contained in Fig. 1, taken a week after the experiment was made. The gelatin with a pH > 5.0 turned dark brown in a few hours when exposed to diffused daylight, while the gelatin with a pH ≷ 4.7 was not affected by light after an exposure of over a week. The photograph also shows that owing to the precipitate in suspension the tubes containing the gelatin at the isoelectric point, pH = 4.6 and 4.7, were more whitish than the rest, which were slightly yellow.

amount of ionic silver in combination with gelatin rises in a regular curve increasing in height with the increase in pH.

20 cc. of each gelatin solution were put into a test-tube and exposed to the light. The five test-tubes that had been treated with M/1024 or less concentrated HCl turned rapidly dark or black in the light, while none of the others did, even when made slightly alkaline and though the exposure lasted for more than a week. The gelatin treated

with 3 M/1024 acid (pH = 4.7) was quite opaque but remained perfectly white; while the test-tube treated with M/256 was only slightly opaque but remained also practically white. This shows that the gelatin treated with 3 M/1024 and whose pH was 4.7 was also entirely free from ionized silver. Fig. 2 is a photograph of these test-tubes. This experiment was repeated several times with the same result. It is a very striking demonstration experiment.

On the more acid side, where the gelatin was free from silver, the gelatin existed as gelatin nitrate as we shall prove presently. This then shows that at the isoelectric point and on the acid side from the isoelectric point a cation cannot combine with gelatin, while on the more alkaline side from the isoelectric point such a combination occurs.

Table I gives the cc. of 0.01 N Ag found in combination with 0.25 gm. of gelatin.

TABLE I.

Cc. 0.01 N Ag in combination with 0.25 gm. of gelatin at different pH.

pH	3.6	3.8	3.8	3.9	4.1	4.3	4.6	4.7	5.0	5.3	5.7	6.1	6.1	6.4
Cc. 0.01 N Ag combined with 0.25 gm. of gelatin	0.5	0.3	0.4	0.3	0.2	0.2	0.2	0.55	1.25	3.2	4.0	4.85	4.6	4.9

The small values of 0.2 cc. or even 0.5 cc. found in the gelatin on the acid side from the isoelectric point are due to incomplete removal by washing; an increase in the number of washings would probably have removed these traces also. Where 0.5 or less cc. of 0.01 N Ag was found in 0.25 gm. of gelatin, light no longer blackened the gelatin. As soon as pH became greater than 4.7 the value of Ag found rose suddenly.

Fig. 3 represents the physical properties of gelatin treated first with M/16 $AgNO_3$ and then brought to different hydrogen ion concentrations by treatment with different concentrations of HNO_3. 1 gm. of powdered gelatin was first put for 30 minutes into 100 cc. of M/16 $AgNO_3$ (pH about 6.9) and the excess of salt was then washed away (in the manner described). Then each gm. of gelatin was perfused three times with a definite concentration of HNO_3 and the excess of acid was washed away by four perfusions with distilled water. The

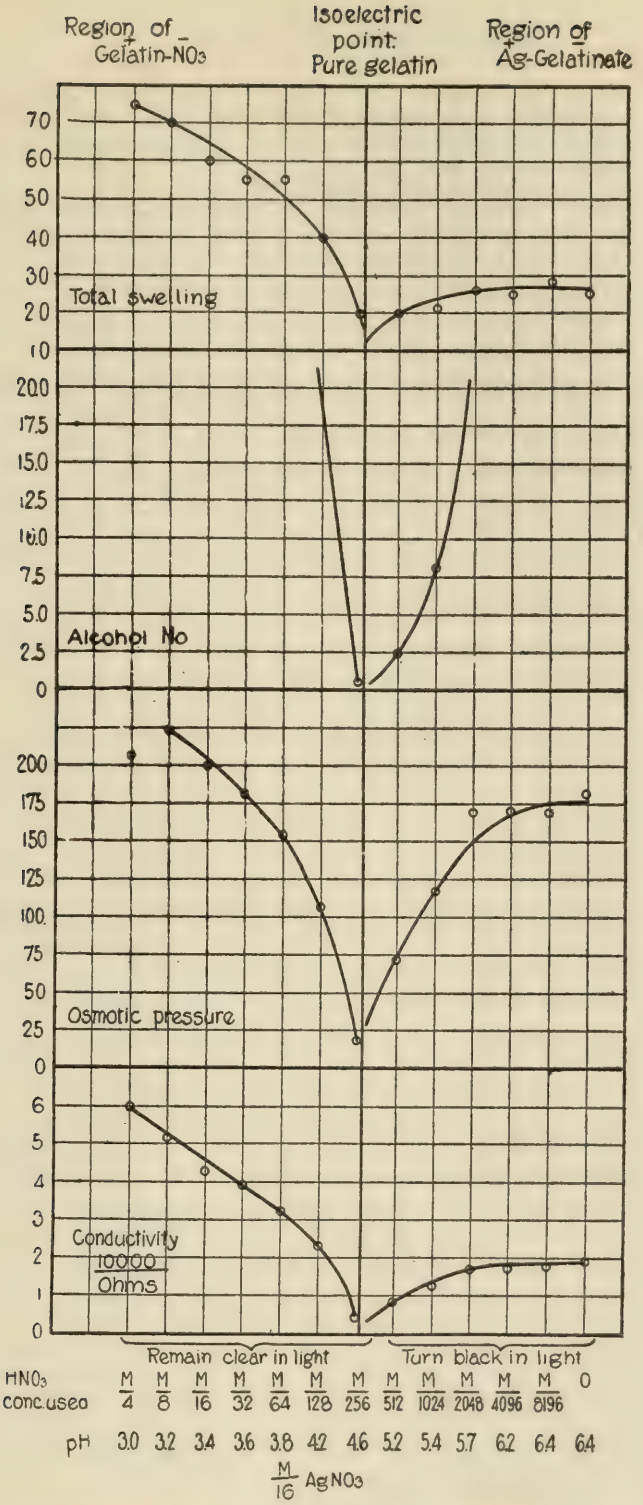


FIG. 3. Gelatin treated first with M/16 $AgNO_3$, washed, then treated with different concentrations of HNO_3, and washed again. Curves for conductivity osmotic pressure, alcohol number, and swelling. On the right from the isoelectric point the gelatin exists as silver gelatinate, on the left as gelatin nitrate, and at the isoelectric point as pure, sparingly soluble gelatin.

swelling of the gelatin was measured while it was in the cylindrical funnel and the height of the column of gelatin in mm. expresses the degree of swelling, all the funnels having the same diameter. The gelatin was then melted, made into a 1 per cent solution, and the conductivity, osmotic pressure (in mm. of the height of the gelatin solution), alcohol number (cc. of 95 per cent alcohol required to cause a definite degree of precipitation in 5 cc. of 1 per cent gelatin solution) were determined. There is an abrupt fall in all the curves near pH 4.6; *i.e.*, at about the isoelectric point, and a rise on both sides. On the right from the isoelectric point (pH > 4.7) the gelatin exists as silver gelatinate, while on the left, more acid side from the isoelectric point (pH < 4.7) the gelatin exists as gelatin nitrate. At the isoelectric point it exists as non-ionized gelatin. This was proved by exposing the gelatin solutions to the light after completion of the experiment which was of course carried out in a dark room; all those gelatin solutions whose pH was from 5.3 to 6.4 turned black when exposed to light, while none of the others was affected by light even after an exposure of more than 2 weeks. Since the original treatment of the gelatin with M/16 $AgNO_3$ near the point of neutrality was the same for all the solutions, it is obvious that the silver gelatinate when its pH was 4.7 or less gave off part if not practically all its silver. This was actually proved to be the case by the analysis of the filtrate of such gelatin during and after the acid treatment.

When we treat the gelatin first with HNO_3, wash the acid away, and then treat the gelatin with M/16 $AgNO_3$ and wash away the excess of salt, we get an identical system of curves and an identical effect of exposure to light, proving that gelatin can only combine with Ag when its pH > 4.7, while it cannot combine with gelatin when its pH $\leqq$ 4.7.

The writer would like to call attention to the fact shown in Figs. 1 and 3 that for pH > 4.7 the curves for swelling and for conductivity are practically parallel to the curve for the amount of silver gelatinate formed.

The Combination of Anions with Gelatin.

In this chapter we intend to furnish the data necessary for proving that gelatin combines with anions only when pH < 4.7, while it can-

not combine or remain in combination with anions when pH $\geqq$ 4.7. We chose for this purpose two anions, Br and CNS, for the volumetric determination of which we possess the convenient Volhard method. Powdered gelatin was first treated with different concentrations of HNO_3 and then washed free from the excess of acid. Then the gelatin in each funnel was perfused three times with 25 cc. of M/8 NaBr and this was followed by six perfusions with 25 cc. of H_2O. The swelling was plotted (upper curve in Fig. 4), the gelatin was melted and made into a 1 per cent solution, and analyzed for Br by the Volhard method. The lower curve gives the quantity of Br in combination with 25 cc. of gelatin. Table II gives the amount of Br found in combination with 0.25 gm. of gelatin. The table, as well as the curve, shows that the treatment of gelatin with M/8 NaBr did not lead to the formation of gelatin bromide when pH $\geqq$ 4.7; while it led to the formation of gelatin Br when pH $<$ 4.7. The quantity of gelatin bromide formed increased with the hydrogen ion concentration.

TABLE II.

Cc. 0.01 N Br in combination with 0.25 gm. of gelatin at different pH.

pH	3.7	3.8	3.9	4.1	4.2	4.6	4.7	5.1	5.8	6.4	6.8	7.0	7.1
Cc. 0.01 N Br combined with 0.25 gm. of gelatin	7.5	7.4	7.1	5.0	2.95	1.4	0.1	0.15	0.1	0.15	0.2	0.1	0.1

In the experiment represented by Fig. 5 everything was the same except that the treatment of gelatin with HNO_3 was followed by three perfusions with M/8 NH_4CNS (instead of M/8 NaBr). The lower curve shows that when pH $\geqq$ 4.7 the gelatin contains no CNS, but that gelatin can combine with CNS as soon as pH $<$ 4.7.

The results are given in Table III. The reader's attention is called to the sharp drop in the values for Br (Table II) as well as for CNS (Table III) at pH = 4.7 and less.

TABLE III.

Cc. 0.01 N CNS in combination with 0.25 gm. of gelatin at different pH.

pH	3.5	3.5	3.9	4.1	4.3	4.4	4.7	4.9	5.5	6.2	6.3	6.4	6.5	7.0
Cc. 0.01 N CNS combined with 0.25 gm. of gelatin	9.4	9.2	6.7	4.1	3.4	1.95	0.15	0.2	0.1	0.1	0.1	0.1	0.1	0

When we reverse the order of treatment, *i.e.* when we treat gelatin first with M/8 NaBr at pH about 7.0, only sodium gelatinate should be formed according to our theory, and no Br should combine with the gelatin. This was found to be correct. When we treat gelatin first

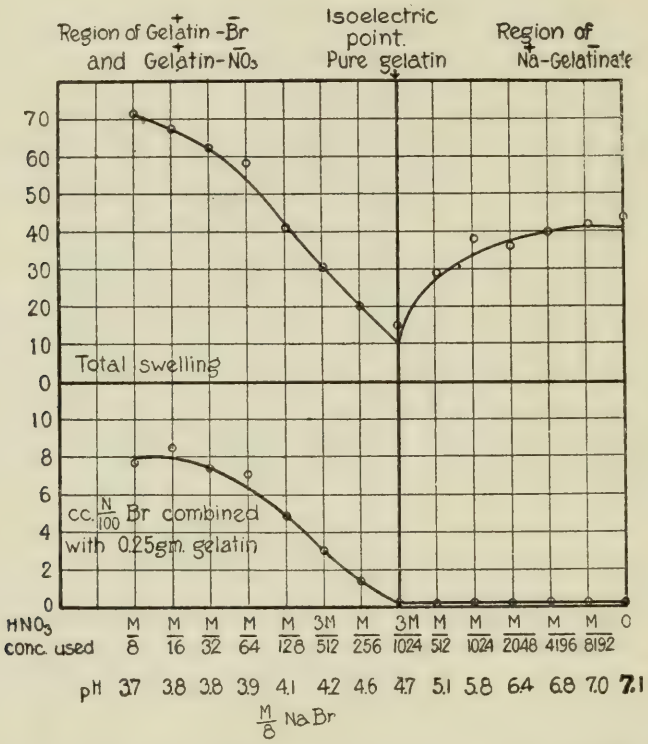

Region of Gelatin-Br and Gelatin-NO3
Isoelectric point. Pure gelatin
Region of Na-Gelatinate
Total swelling
cc. N/100 Br combined with 0.25gm. gelatin
HNO3 conc. used
M/8 M/16 M/32 M/64 M/128 3M/512 M/256 3M/1024 M/512 M/1024 M/2048 M/4196 M/8192 0
pH 3.7 3.8 3.8 3.9 4.1 4.2 4.6 4.7 5.1 5.8 6.4 6.8 7.0 7.1
M/8 NaBr

FIG. 4. Gelatin treated with different concentrations of HNO_3, washed, treated with M/8 NaBr, and washed again. The ordinates of the lower curve are the values of Br in combination with gelatin, showing that on the right (alkaline) side from the isoelectric point and at the isoelectric point gelatin contains no Br, while on the left, more acid side from the isoelectric point, the amount of the Br found increases with the pH. The ordinates of the upper curve represent the swelling of the same gelatin. The two curves on the left side are almost parallel, showing that the degree of swelling is determined by the relative mass of the gelatin bromide (or nitrate) formed.

with M/8 NaBr, then with varying concentrations of HNO_3, no Br should enter into ionogenic combination with the gelatin. This is confirmed by the volumetric analysis which shows that such gelatin is free from ionic Br. Only sodium gelatinate is formed under these conditions.

Figs. 4 and 5 confirm a fact already referred to in connection with Fig. 1. The curves for the swelling of gelatin for pH < 4.7 are parallel to the curves representing the amount of gelatin Br and gelatin CNS formed. Fig. 6 gives the relation of the curve for swelling to the curves for the other physical properties of gelatin treated with acid and then with M/8 NaBr. These experiments prove definitely that on the acid side from the isoelectric point gelatin (and probably amphoteric colloids in general) can combine only with anions, on the

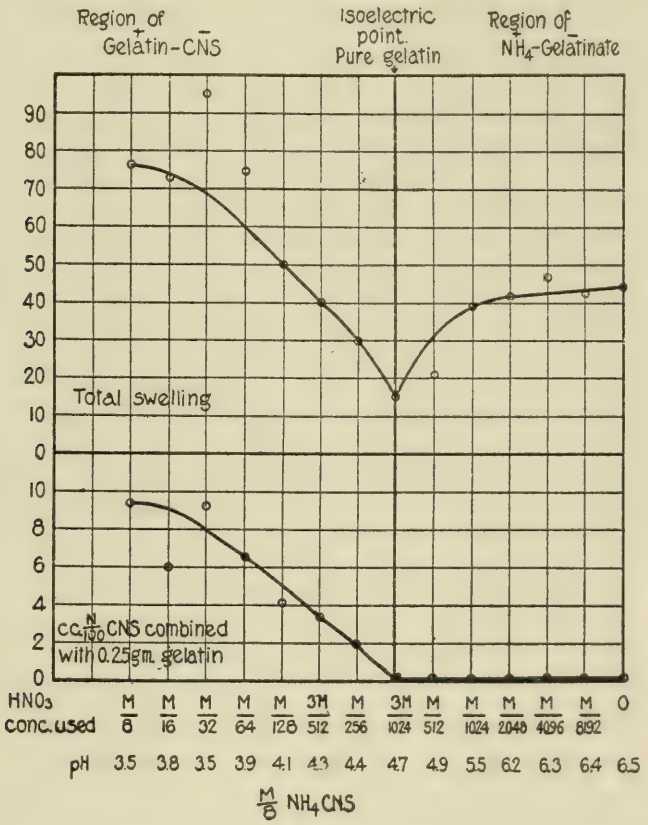

Region of Gelatin-CNS
Isoelectric point. Pure gelatin
Region of NH4-Gelatinate
Total swelling
cc. N/100 CNS combined with 0.25gm. gelatin
HNO3 conc. used
M/8 M/16 M/32 M/64 M/128 3M/512 M/256 3M/1024 M/512 M/1024 M/2048 M/4096 M/8192 0
pH 3.5 3.8 3.5 3.9 4.1 4.3 4.4 4.7 4.9 5.5 6.2 6.3 6.4 6.5
M/8 NH4CNS

FIG. 5. Gelatin treated with different concentrations of HNO_3, washed, then treated with M/8 NH_4CNS, and washed again. Further explanation of figure corresponds to that for Fig. 4.

more alkaline side from the isoelectric point only with cations, and at the isoelectric point with neither.

Action of Heavy Metals and Polyvalent Ions.

When we treat finely powdered gelatin with a solution of M/10 copper acetate, the powder assumes a blue color due to the formation

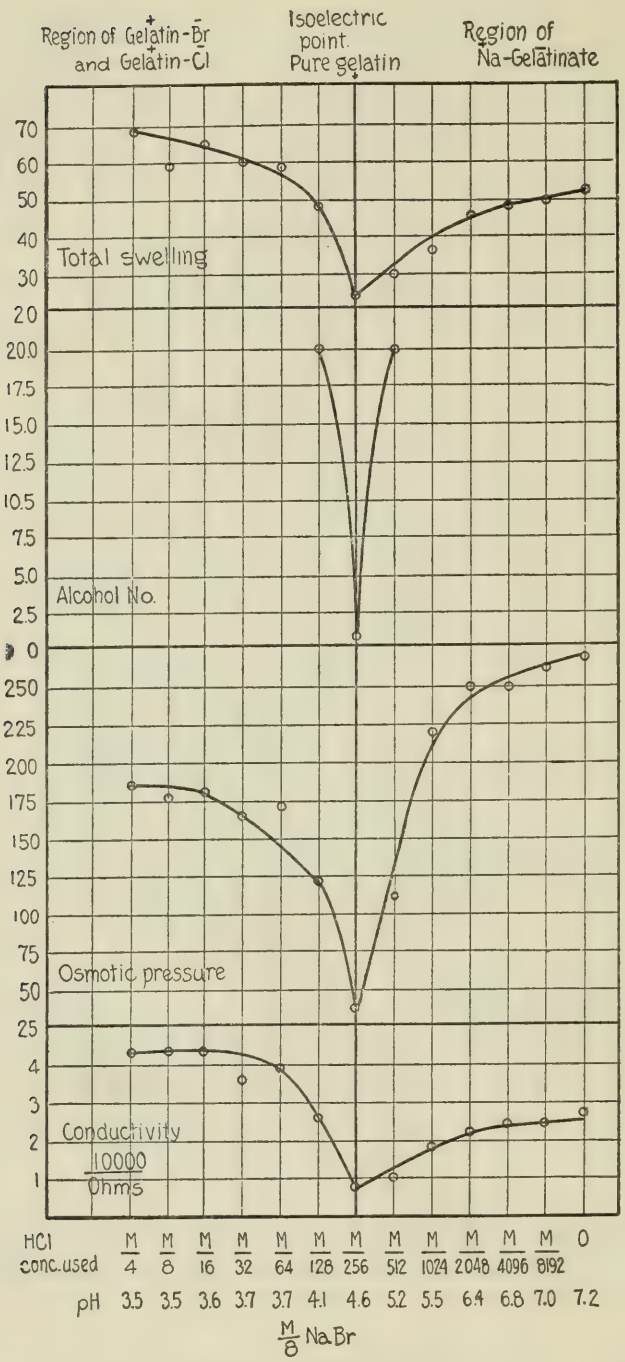

Region of Gelatin-Br and Gelatin-Cl
Isoelectric point. Pure gelatin
Region of Na-Gelatinate
Total swelling
Alcohol No.
Osmotic pressure
Conductivity 10000/Ohms
HCl conc. used
M/4 M/8 M/16 M/32 M/64 M/128 M/256 M/512 M/1024 M/2048 M/4096 M/8192 0
pH 3.5 3.5 3.6 3.7 3.7 4.1 4.6 5.2 5.5 6.4 6.8 7.0 7.2
M/8 NaBr

FIG. 6. Gelatin treated first with varying concentrations of HCl, washed, then treated with M/8 NaBr, and washed again. Curves for conductivity, osmotic pressure, alcohol number, and swelling. On the right side from the isoelectric point gelatin exists as sodium gelatinate, on the left side as gelatin chloride or bromide, at the isoelectric point as pure, sparingly soluble gelatin.

of copper gelatinate (pH being about 6.3). When we wash off the excess of copper acetate solution, the blue color remains. When we treat such copper gelatinate with an acid to increase the hydrogen ion concentration, we notice that the gelatin loses its blue color as soon as its pH $\lessapprox$ 4.7. If after the acid treatment gelatin is perfused six times with distilled water, the gelatin keeps its blue color when pH $>$ 4.7, and it is possible to demonstrate the presence of ionized copper in such gelatin with the ferrocyanide test. When the pH of the gelatin is $\lessapprox$ 4.7, the ferrocyanide test shows that the gelatin is free from ionized copper. The water with which this gelatin was washed contains the copper, while the water with which the gelatin with a pH $>$ 4.7 was washed is free from copper, except that which was originally contained in the capillary spaces between the granules of gelatin and which was carried away with the first perfusions.

Fig. 7 gives the curves of gelatin first treated for 30 minutes with M/16 copper acetate and subsequently with from M/8 to M/8192 HCl. For pH $\gtrapprox$ 4.6 all the values of the curves are as low as they are at the isoelectric point, and for the same reason; namely, copper gelatinate as well as the gelatin at the isoelectric point being sparingly soluble. (It is well to keep in mind that at the isoelectric point the gelatin contains no ionizable copper, while for pH $>$ 4.7, the gelatin is in combination with ionic copper.) On the left, more acid side from the isoelectric point the gelatin exists in the form of gelatin chloride and gelatin acetate.

The experiment proves, therefore, that copper gelatinate cannot continue to exist when pH $\lessapprox$ 4.7, and that the gelatin gives off its copper at such pH.

The same experiment was made with $CeCl_3$ and Pb acetate$_4$, and almost identical curves were obtained as in the case of copper acetate, Ce as well as Pb forming sparingly soluble gelatinates. In the case of K_4FeCN_6 the sparingly soluble gelatin$_4$ $FeCN_6$ was formed only on the acid side of the isoelectric point as our theory demands. Fig. 8 gives the curves. The gelatin was first treated with different concentrations of HCl and then with M/16 K_4FeCN_6 in the manner described for the other cases.

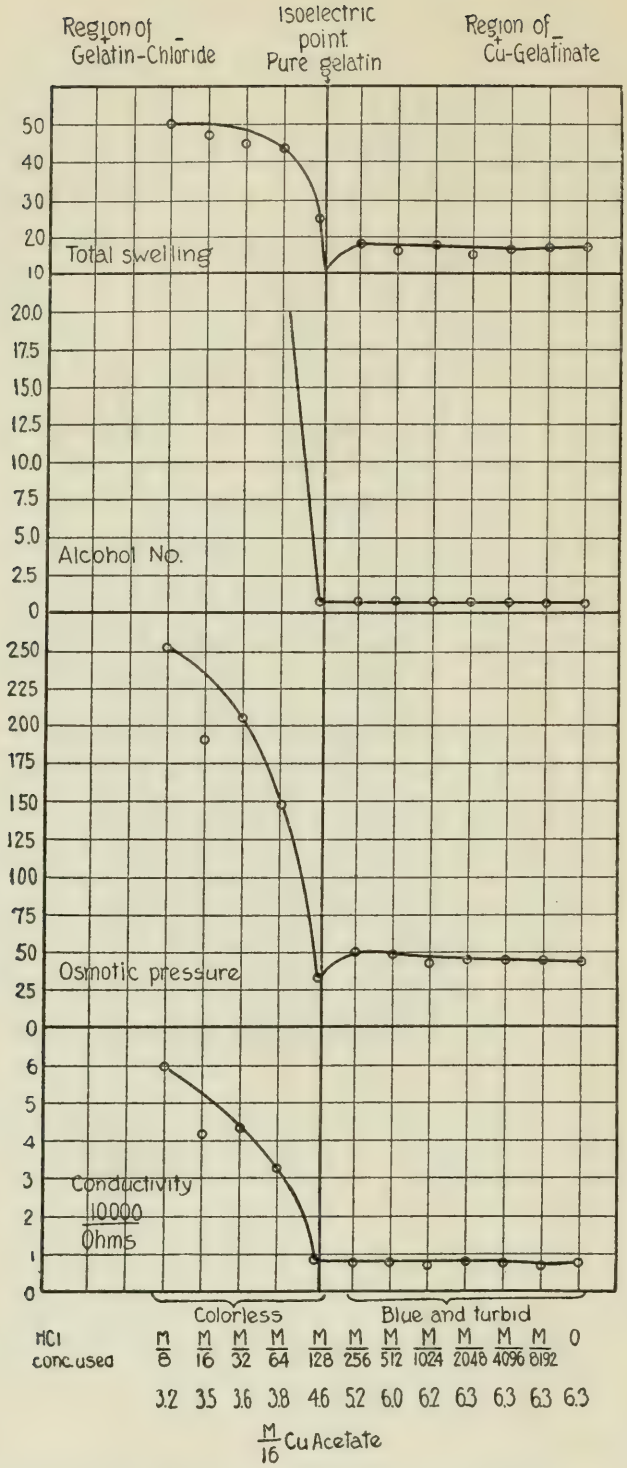

Region of + Gelatin-Chloride −
Isoelectric point Pure gelatin
Region of − Cu-Gelatinate +
Total swelling
Alcohol No.
Osmotic pressure
Conductivity 10000/Ohms
Colorless
Blue and turbid
HCl conc. used
M/8 M/16 M/32 M/64 M/128 M/256 M/512 M/1024 M/2048 M/4096 M/8192 0
3.2 3.5 3.6 3.8 4.6 5.2 6.0 6.2 6.3 6.3 6.3 6.3
M/16 Cu Acetate

FIG. 7. Gelatin treated with M/16 copper acetate, washed, treated with different concentrations of HCl, and washed again. On the right side from the isoelectric point gelatin exists as copper gelatinate, stained blue, practically insoluble, and with low conductivity, osmotic pressure, alcohol number, and swelling. On the left side from the isoelectric point it exists as gelatin chloride (and acetate), highly soluble, and with high conductivity, osmotic pressure, alcohol number, ands welling; at the isoelectric point pure gelatin, practically insoluble.

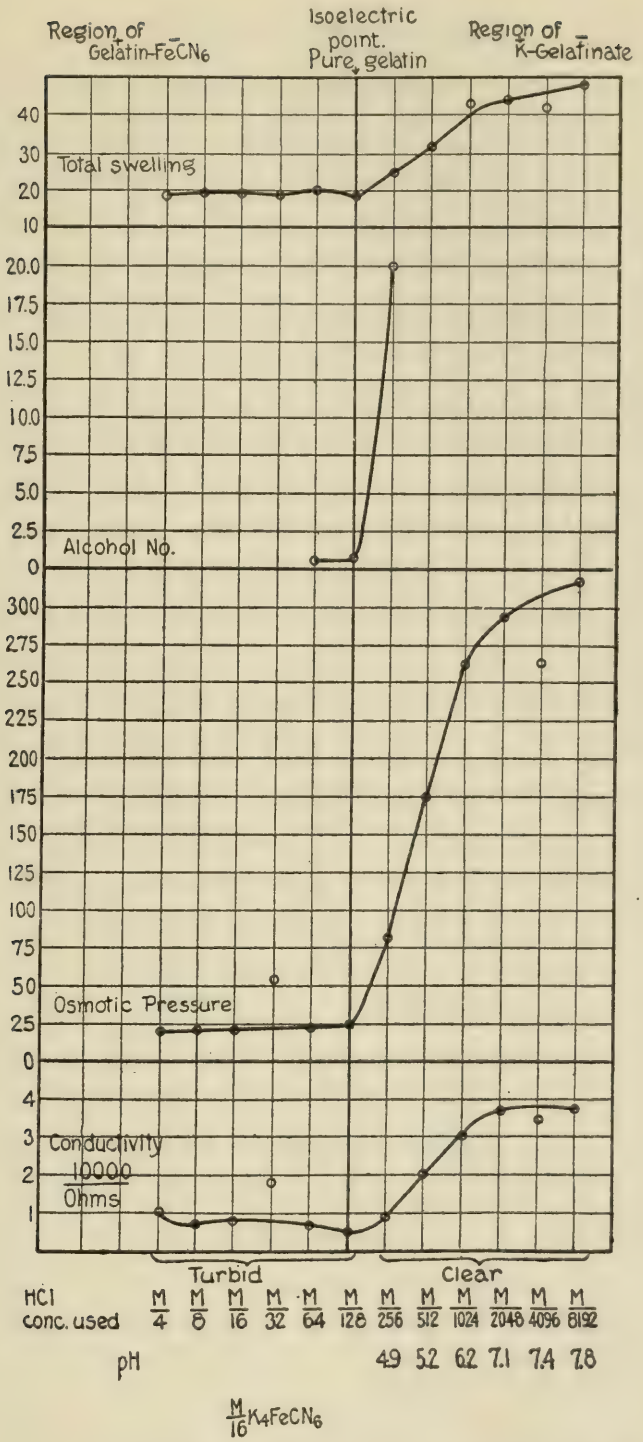


FIG. 8. Gelatin treated with different concentrations of HCl, washed, then treated with M/16 K_4FeCN_6, and then washed again. On the left, more acid side from the isoelectric point, gelatin exists as insoluble gelatin ferrocyanide, with low osmotic pressure, low conductivity, etc.; on the right, more alkaline side from the isoelectric point, gelatin exists as potassium gelatinate with high conductivity, osmotic pressure, etc.; at the isoelectric point, pure and sparingly soluble gelatin.

Experiments with Dyes.

Gelatin is readily stained with the basic dye neutral red at the point of neutrality. Our theory demands that gelatin thus stained with neutral red should give off all its stain at the isoelectric point and on the acid side from the isoelectric point; while the neutral red should be retained when pH > 4.7. This turns out as the theory demands. 1 gm. of finely powdered gelatin was put for 20 minutes at about 15°C. into a series of beakers each containing 1 cc. of M/100 neutral red. The gelatin was then filtered and washed twice with 25 cc. of distilled water. All the gelatin was stained a beautiful deep red. Each funnel was then perfused three times with a definite concentration of HCl, the concentration for the various funnels varying from M/8 to M/8192, and this was followed by eight perfusions with 25 cc. of H_2O each. In the funnels treated with a concentration of HCl of M/512 or above, the gelatin became decolorized (the neutral red being washed away); while all the funnels treated with acid of a lower concentration retained their deep red color and did not give off their stain. The decolorized gelatin was then melted, made into a 1 per cent solution whose pH was determined. It was found that the gelatin previously treated with M/512 HCl and decolorized had a pH = 4.7, that treated with 3 M/1024 having a pH = 4.6, and so on. Hence the gelatin was not able to retain its neutral red when brought to the isoelectric point or to a pH $\leqq$ 4.7, as our theory demands.

Michaelis and Davidsohn[3] working with large blocks of gelatin, gained the impression that gelatin is slightly stainable with both basic and acid stains at the isoelectric point. We are inclined to believe that our method of staining gelatin in a finely divided condition and then ascertaining the pH where the dye is given off again by the gelatin, gives more reliable results than their method of working with large blocks of gelatin. We intend to return to the problem of staining in a future communication.

SUMMARY.

1. It is shown by volumetric analysis that on the alkaline side from its isoelectric point gelatin combines with cations only, but not

[3] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1913, liv, 323.

with anions; that on the more acid side from its isoelectric point it combines only with anions but not with cations; and that at the isoelectric point, pH = 4.7, it combines with neither anion nor cation This confirms our statement made in a previous paper that gelatin can exist only as an anion on the alkaline side from its isoelectric point and only as a cation on the more acid side of its isoelectric point, and practically as neither anion nor cation at the isoelectric point.

2. Since at the isoelectric point gelatin (and probably amphoteric colloids generally) must give off any ion with which it was combined, the simplest method of obtaining amphoteric colloids approximately free from ionogenic impurities would seem to consist in bringing them to the hydrogen ion concentration characteristic of their isoelectric point (*i.e.*, at which they migrate neither to the cathode nor anode of an electric field).

3. It is shown by volumetric analysis that when gelatin is in combination with a monovalent ion (Ag, Br, CNS), the curve representing the amount of ion-gelatin formed is approximately parallel to the curve for swelling, osmotic pressure, and viscosity. This fact proves that the influence of ions upon these properties is determined by the chemical or stoichiometrical and not by the "colloidal" condition of gelatin.

4. The sharp drop of these curves at the isoelectric point finds its explanation in an equal drop of the water solubility of pure gelatin, which is proved by the formation of a precipitate. It is not yet possible to state whether this drop of the solubility is merely due to lack of ionization of the gelatin or also to the formation of an insoluble tautomeric or polymeric compound of gelatin at the isoelectric point.

5. On account of this sudden drop slight changes in the hydrogen ion concentration have a considerably greater chemical and physical effect in the region of the isoelectric point than at some distance from this point. This fact may be of biological significance since a number of amphoteric colloids in the body seem to have their isoelectric point inside the range of the normal variation of the hydrogen ion concentration of blood, lymph, or cell sap.

6. Our experiments show that while a slight change in the hydrogen

ion concentration increases the water solubility of gelatin near the isoelectric point, no increase in the solubility can be produced by treating gelatin at the isoelectric point with any other kind of monovalent or polyvalent ion; a fact apparently not in harmony with the adsorption theory of colloids, but in harmony with a chemical conception of proteins.

The writer wishes to express his thanks to Mr. M. Kunitz for his faithful assistance in these experiments.

[Reprinted from The Journal of Biological Chemistry, August, 1918, Vol. xxxv, No. 2, pp. 179–219.]

A STUDY ON AUTOLYSIS OF ANIMAL TISSUES.

By KARL GUSTAV DERNBY.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, May 1, 1918.)

CONTENTS.

Introduction 359
General methods 363
Preparation of tissue suspensions 363
Nitrogen determinations 363
Determination of the hydrogen ion concentration 364
Gelatin method 365
Peptone method 366
Artificial autolysis 367
Identification of enzymes 370
Yeast cells 370
Liver 370
Spleen 375
Pancreas 377
Stomach 382
Leukocytes 385
Autolysis 387
Liver 387
Spleen 391
Pancreas 393
Influence of neutral salts on animal enzymes 396
Summary 397
Bibliography 399

INTRODUCTION.

By autolysis we mean the non-bacterial postmortem self-decomposition of animal or plant tissues. Salkowski (1890) first recognized the phenomenon as due to enzymatic action, and called it "autodigestion." The name "autolysis" was introduced later by Jacobi (1900). The many papers published on autolysis have pursued two different lines of investigation, one concerning the products of autol-

ysis, the other concerning the action and nature of the proteolytic enzymes causing the autolysis.

Through the work of authors such as Salkowski, E. Fischer, Wroblewski, Kossel, Kutscher, Levene, and others on the problem of the products of autolysis, we know that the end-products of tissue decomposition are amino-acids and intermediate products, albumoses, peptones, and peptides, such as are obtained by the action of pepsin, trypsin, and erepsin. But the nature and manner of action of the different proteolytic enzymes is still a matter of discussion, and the question is one of biological importance, because it appears probable that these autolytic enzymes are not postmortem products, but the very enzymes which act during the life of the cells.

The literature concerning the mode of action of these enzymes is a maze of contradictions. The most exhaustively studied factor is the influence exerted by acids and bases on autolysis. Early investigators as Schwiening (1894) and Biondi (1896) stated that alkalies check and acids facilitate autolysis. Hedin and Rowland (1901) demonstrated the presence of proteases in the press juices of animal tissues, obtained by the Buchner method, and state that alkaline reaction depresses autolysis, and that acids in weak concentrations facilitate it, the rate of autolysis being greatest in the presence of 0.25 per cent acetic acid. Hedin (1906) especially called attention to what he termed "α-protease," a proteolytic enzyme acting in alkaline solution and distinctly different from "β-protease," which acts only in acid reaction. Hedin also made the interesting observation, that if a tissue such as liver or spleen is pre-treated with dilute acids and then transferred into alkaline solution, the rate of autolysis in alkaline medium is much greater than if the tissue is immersed in the alkaline solution without preliminary acid treatment. In order to explain this phenomenon he assumes the existence of "antiproteases," which inhibit only the α-protease and are readily destroyed in acid solution.

A similar theory for the autolysis of yeast is proposed by Buchner and Hahn (1909, 1910) who assume that there are "antiproteases, probably of lipoid character," which protect the proteins from being destroyed by the proteolytic enzymes. Satisfactory proof of the existence of such antienzymes, seems, however, to be lacking.

Other authors besides Hedin have studied the antiproteolytic actions of serum and different tissues; *e.g.*, Weinland (1903), Landsteiner (1900), and Opie (1905, 1906). Attention may also be called to the work of Levene and Stookey (1903) in this respect. Levene (1905) has given an exhaustive review of all data concerning autolysis, known up to that date. As has been said, most authors have found that alkalies inhibit autolysis, but there are others such as Baer and Loeb (1905) who state that considerable autolysis occurs in alkaline medium. Opie (1905, 1906) and later Dochez (1910) have published a series of papers concerning autolysis of various organs. Dochez contributes valuable data on liver autolysis, confirming the observation of Hedin and others, and gives a good historical review of the field.

More recently some other American authors, especially Bradley (1915, 1916) and collaborators and Morse (1917) have published papers on autolysis, and on the influence thereon of the reaction of the medium. Morse assumes autolysis to be an "autocatalytic" reaction, due to the spontaneous increase in the hydrogen ion concentration. Bradley explains the accelerating influence of acid upon autolysis by assuming that the increased hydrogen ion concentration alters the proteins into forms more readily digested by the enzymes. Bradley also makes the statement, that there probably is no "latent period" in the autolysis, and that the first stage of autolysis is the formation of peptones and peptides, amino-acids appearing only later.

As far as the author knows, however, there have been no other attempts made with modern methods to study the influence of the hydrogen ion concentration on autolysis of animal tissues.

In his classical work Sörensen (1909) emphasizes that it is quite as important to determine the hydrogen ion concentration and its eventual changes as the temperature of biochemical processes. Thanks to him we now have accurate methods to measure hydrogen ion concentration. In this connection attention must be called to the theory, first suggested by Loeb and later adopted by Michaelis (1914), that the enzymes in fact are ampholytes and that enzymatic action is a dissociation phenomenon. Ringer (1916) does not accept this theory.

In a previous paper the author has studied the proteolytic enzymes in yeast cells and the effect of hydrogen ion concentration on them. Briefly stated this problem has been studied in the following manner. Buchner and Hahn (1903) who discovered cell-free fermentation, also studied the proteolytic activity of the press juice, and claimed that this contained an enzyme of trypsin type, which, however, acted only in acid solution, and to which they gave the name "endotryptase." They described it as acting best in the presence of 0.2 per cent hydrochloric acid. There have been a great number of papers dealing with this problem, mostly confirming the work of Hahn. The English botanist, Vines (1904, 1909), however, claims that in the yeast cells there are two different kinds of proteolytic enzymes, one of pepsin, and one of trypsin type, and that they differ in solubility in sodium chloride solution.

The writer (1917) has been able to show, that in yeast cells there must be at least three different groups of proteolytic enzymes, acting at different hydrogen ion concentrations (Fig. 1).

	Action.	Optimum hydrogen ion concentration.
		pH
Pepsin	Splitting proteins into peptones.	About 4
Tryptase	" peptones " peptides.	7.0
Ereptase	" peptides " amino-acids.	7.8

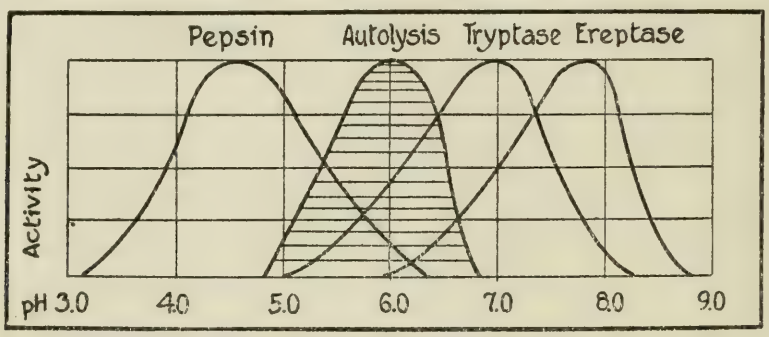


FIG. 1.

The optimum for the action of pepsin lies in the acid region, that for the tryptase almost at the neutral point and that for the ereptase in alkaline solution. The optimum for autolysis (the maximum

decomposition of the protein) at constant hydrogen ion concentration is however at pH = 6, between the optima of pepsin and tryptase, and at the point where the two enzymes can act simultaneously. The extent of autolysis is shown in the shaded portion of the figure (Fig. 1).

In the following the author has extended the investigations to different tissues of animal origin, always controlling the hydrogen ion concentration of the medium and its changes.

General Methods.

Preparation of Tissue Suspensions.—The tissues used in the following experiments were obtained from freshly killed normal animals, dogs or pigs. The liver, pancreas, and spleen tissues, weighing from 50 to 300 gm. and freed mechanically from attaching fat, were minced in a meat chopper with an ordinary cutter, repeatedly washed with 1 per cent sodium chloride solution to remove most of the blood, and filtered through cheese-cloth. The remaining mash was then minced with a "nut butter cutter" until it became a very fine homogeneous suspension which could be easily pipetted and contained about twice as much water as the original tissue. How much of these suspensions was used in each experiment, and their content of nitrogen is noted under each experiment. These suspensions were kept in the ice box, and toluene and chloroform in sufficient quantities were added to prevent bacterial growth. All the digestions were carried out in an incubator at 37°C.

Nitrogen Determinations.—The chemical methods used for showing the progress of the enzymatic reactions were the following. The total amount of nitrogen in the solutions was determined by the Kjeldahl method. The coagulable protein was precipitated with 2.5 per cent trichloroacetic acid (9 parts of acid to 1 part of solution). After removing the coagulum the remaining nitrogen in an aliquot part of the filtrate was determined by the Kjeldahl method. The amount of amino nitrogen was determined by the Van Slyke method[1]

[1] In an earlier paper the author used Sörensen's formol titration for determining the amount of amino nitrogen liberated. This method measures practically all NH_2 groups, but it requires rather large amounts of solution for each test to

in the solution after removing the solid particles by filtration. The peptide nitrogen, that is the nitrogen from the – CO – NH – group of albumoses, peptides and peptones, was determined by hydrolyzing the solutions with hydrochloric acid. 1 part of concentrated hydrochloric acid was added to 1 part of solution. The mixture was heated for 24 hours at 100°, concentrated in a vacuum to remove the acid, and finally neutralized with sodium hydroxide and made up to the original volume of the amino-acid solution. Then the increase in amino nitrogen was determined by the Van Slyke method. The free ammonia was determined by addition of potassium carbonate and aeration into 0.02 M hydrochloric acid.

Determination of the Hydrogen Ion Concentration.—The hydrogen ion concentrations in all the experiments were determined by the colorimetric method of Sörensen with the indicators of Clark and Lubs (1917). Because of their small "protein error" these new indicators are well fitted for solutions containing protein substances. To express the value of the hydrogen ion concentration Sörensen's symbol pH is used. pH indicates the negative logarithm of the hydrogen ion concentration in terms of normality.

The standard solutions were made up of pure chemicals, and their purity was controlled by the electrometric method. The following solutions were used:

$\frac{1}{10}$ M hydrochloric acid.
$\frac{1}{10}$ " sodium citrate.
$\frac{1}{15}$ " potassium dihydrogen phosphate, KH_2PO_4.
$\frac{1}{15}$ " disodium hydrogen phosphate, $Na_2HPO_4.2H_2O$.

The last two salts were Kahlbaum's preparations after Sörensen. By means of mixtures of these solutions any hydrogen ion concentration between pH = 1.0 and pH = 9.0 can be reproduced, and they cover practically the whole range of the hydrogen ion concentration which can be considered in the study of autolysis.

The indicators used have been the following.

get accurate values. The Van Slyke method does not give the accurate amount of nitrogen for some substances, *e.g.* ammonia and glycyl derivatives, but has the great advantage that carbon dioxide and phosphates do not interfere with it, and rather small amounts of solutions are required for each test.

	Range of pH.
Thymolsulfonephthalein (thymol blue)	1.2–2.8
Tetrabromophenolsulfonephthalein	3.0–4.6
Methyl red	4.4–6.0
Dibromo-*o*-cresolsulfonephthalein	5.2–6.8
Dibromothymolsulfonephthalein	6.0–7.6
Phenolsulfonephthalein	6.8–8.4
o-Cresolsulfonephthalein	7.2–8.8
Thymolsulfonephthalein	8.0–9.6
o-Cresolphthalein	8.2–9.8

As most of the solutions examined were more or less colored and turbid, they have been diluted two or three times before the examination. According to Clark such a dilution does not essentially alter the hydrogen ion concentration of the solutions. Clark also suggests the use of a special comparator rack for colored solutions. It has been used here in a slightly modified shape and found to work excellently. In another paper is a complete description of it (Avery and Dernby, 1918). The method is fairly accurate; the experimental error averages less than 0.1 in pH.

For the identification of the different proteolytic enzymes in the tissues the following two methods have proved to give the best results, and to be the most convenient to carry out.

Gelatin Method.—The method of Fermi (1891, 1906) of liquefying gelatin in the form suggested by Palitzsch and Walbum (1912) has been used to demonstrate the presence of pepsin-like enzymes. In some cases also enzymes of trypsin type attack gelatin. As this method does not seem to be generally known, its main features may be described here. 700 gm. of gelatin are dissolved in 1,250 cc. of hot water, the solution is passed through cheese-cloth, and 2 gm. of thymol suspended in water are added. The solution is then diluted to 2 liters and kept in stock in an ice box. As used in the following experiments it contained 37.5 mg. of nitrogen per 1 cc.

Before the experiments, 200 gm. of the stock solution were taken and diluted to 500 cc. For most of the experiments 15 cc. of this solution were used, hydrochloric acid, sodium hydroxide, phosphates, and tissue suspension added, and the whole was diluted to 30 cc. The solutions were kept in small Erlenmeyer flasks, covered with an inverted beaker, and all the digestions took place in an incubator at

37°. After certain intervals 5 cc. from each flask were removed and pipetted into a thin test-tube, which subsequently was dipped into ice water and cooled for exactly 15 minutes. Acids in less than M concentration do not interfere much with the liquefaction of gelatin, but even comparatively small amounts of alkalies do. Therefore the alkaline solutions must be neutralized by addition of a calculated amount of acid before samples are removed for cooling. After 15 minutes the tubes were removed from the ice bath, and the degree of liquefaction was estimated.

In order to make possible a graphic representation of the results in curves and to facilitate their presentation, the estimate is expressed in numbers. Palitzsch and Walbum utilize twelve numbers, out of which, however, in this paper only seven are used. They are the following:

0 = Completely solid.
1 = Solid, but small pieces may be torn off by strong shaking.
2 = " " the surface moves somewhat when tubes are shaken.
3 = Soft.
4 = Half liquid.
5 = Almost "
6 = Entirely "

Peptone Method.—For the demonstration of trypsin- or erepsin-like enzymes solutions of Witte's peptone have been used. A 4 per cent solution of Witte's peptone was made up (Dernby, 1917) and used as stock solution. As used here it contained 7.92 mg. of total nitrogen per 1 cc. In order to carry out the digestions with different tissues under identical conditions seven different mixtures of peptone, phosphates, sodium hydroxide or hydrochloric acid, and water were prepared beforehand, all possessing different hydrogen ion concentrations. The following scheme shows the composition of the mixtures and the resulting hydrogen ion concentration.

An experiment was carried out in the following manner. In seven test-tubes were transferred 9 cc. of each of these solutions, and to each of them was then added 1 cc. of the enzyme preparation or tissue suspension. Care must be taken that the tissue suspension does not essentially alter the hydrogen ion concentration of the solutions. In some cases a few drops of hydrochloric acid or sodium

40 cc. 4 per cent peptone solution + 10 cc. 0.5 M phosphates + HCl or NaOH + water = 180 cc.

No.	0.1 N HCl.	0.1 N NaOH.	0.5 M phosphates.		pH
			KH_2PO_4	Na_2HPO_4	
	cc.	*cc.*	*cc.*	*cc.*	
1	16.0	—	10.0	0	4.0
2	8.0	—	9.0	1.0	5.2
3	3.0	—	5.0	5.0	6.8
4	—	—	2.0	8.0	7.3
5	—	3.0	1.0	9.0	7.7
6	—	8.0	0.5	9.5	8.0
7	—	14.0	0	10.0	8.5

hydroxide had to be added beforehand in order to keep the initial hydrogen ion concentration at the desired point.

At the beginning and after certain intervals the amino nitrogen and the hydrogen ion concentration were determined as described above.

Artificial Autolysis.

In explanation of the autolysis of yeast we have suggested that there are enzymes in the cells of peptic, tryptic, and ereptic character, all of them working at different hydrogen ion concentrations, and "autolysis" is the resultant of the action of these different enzymes. To test this theory more completely it appears desirable to carry out an "artificial autolysis," that is to treat a known protein substance with a mixture of pepsin and trypsin, and analyze the reaction products after digestion at different hydrogen ion concentrations. In order to do this the following experiment was carried out by means of the method of Palitzsch and Walbum (1912) with gelatin as substrate. The gelatin solutions prepared as above were treated with certain amounts of hydrochloric acid or sodium hydroxide in order to obtain a wide range of different hydrogen ion concentrations. A solution containing both pepsin and trypsin was made by dissolving a 0.1 gm. portion of each enzyme (Fairchild) in 50 cc. of distilled water. Of this solution 1.0 cc. was added to 30 cc. of gelatin mixture. No buffer mixture was used, the initial hydrogen ion concentration was measured as stated, and if it changed a little dur-

ing the digestion it was of no essential importance. Table I and Fig. 2 show the result.

TABLE I.

Trypsin and Pepsin on Gelatin.

15 cc. gelatin + HCl or NaOH + 1.0 cc. enzyme solution + water = 30 cc.
Enzyme solution: 0.1 gm. pepsin (Fairchild) + 0.1 gm. trypsin (Fairchild) + 50 cc. water.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for				
	HCl	NaOH		30 min.	60 min.	120 min.	180 min.	360 min.
1	6.0 cc. N	—	2.0	1½	1½	2	2½	3
2	4.0 " "	—	2.5	1½	3	3½	4	5
3	3.0 " "	—	3.1	1	3	3½	4	5
4	1.6 " "	—	3.5	½	2	2½	3	4
5	6.0 " 0.1 N	—	4.1	0	½	1	2	5
6	—	—	4.9	0	0	0	1	6
7	—	3.0 cc. 0.1 N	6.0	0	0	1	3	6
8	—	4.0 " 0.1 "	6.8	0	1	2	5	6
9	—	6.0 " 0.1 "	7.3	0	2	4	6	6
10	—	7.0 " 0.1 "	7.7	½	3	5	6	6
11	—	8.0 " 0.1 "	8.1	½	3	5	6	6
12	—	1.5 " N	8.6	½	2	4	6	6

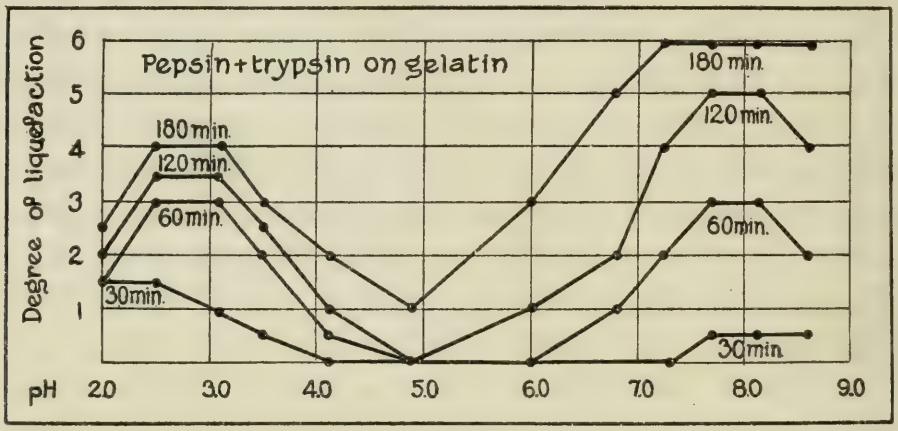


FIG. 2.

The gelatin is liquefied in two regions, one on the acid side, the other on the alkaline. There are two well defined optima, one at about pH = 3 and the other at about pH = 8. It is obvious that

the first optimum must be due to the action of the pepsin, the other to the action of the trypsin.

In the above experiment we have used a substrate, gelatin, which is attacked by both pepsin and trypsin. Most native animal proteins, are attacked by pepsin but not by trypsin, the latter enzyme acting only on the products of peptic digestion. We must expect results quite different from the above when a substrate not attacked directly by trypsin is acted on by the two enzymes.

TABLE II.

Trypsin and Pepsin on Liver Proteins.

2 cc. boiled liver suspension + 1 cc. 0.5 N phosphates + HCl or NaOH + 1 cc. enzyme + water = 10 cc.

2 cc. suspension contained 23.6 mg. total nitrogen.

Enzyme solution = 0.1 gm. pepsin (Fairchild) + 0.1 gm. trypsin in 50 cc. water.

Digestion continued 12 hours at 37°.

Tube No.	Initial pH.	Increase in amino nitrogen.
		mg.
1	About 2	1.55
2	3.5	1.67
3	4.5	1.70
4	5.5	2.80
5	6.4	3 20
6	7.0	2.76
7	7.5	2.68
8	8.0	2.46

Table II gives an experiment where boiled pig's liver, thoroughly minced and washed, is used as such a substrate.

The optimum for the reaction is about pH = 5 to 6. This pH lies between the optima of pepsin and trypsin, but is where both enzymes can act simultaneously. On the alkaline side of the optimum there has been more digestion than on the acid side, presumably because the liver contains some proteins digestible directly by trypsin. These results are by no means to be regarded as complete evidence for the general theory of autolysis developed in the following, but they prove that it is easy by known enzymes and substrates to reproduce a process resembling autolysis.

Identification of the Enzymes.

The classification of proteolytic enzymes hitherto used is made on three lines. (1) The substrate which they attack. (2) The products yielded. (3) The optimum hydrogen ion concentration for the enzyme action.

As the classical types for the proteolytic enzymes are the three excreted enzymes from the animal body, the pepsin, the trypsin, and the erepsin, the scheme for their actions is the following:

Enzyme.	Substrate.	Products.	Optimum pH.
Pepsin.....	Native proteins.	Peptones.	1.5
Trypsin.....	Albumoses, peptones, and some native proteins, such as casein and fibrin.	Peptides and amino-acids.	7.8
Erepsin.....	Peptides.	Amino-acids.	7.8

Since it is difficult, if not impossible, to separate the proteolytic enzymes from the tissue cells, a suspension of very finely minced tissue is used in the following as enzyme preparation. As substrates gelatin solutions have been used for detecting enzymes of pepsin type, and peptone solutions for detecting enzymes of trypsin or erepsin type. Briefly, those two tests are called the "gelatin test" and the "peptone test."

Yeast Cells.—In an earlier paper it is shown that the existence of both pepsin- and trypsin-like enzymes can be easily demonstrated by the action of yeast juice on gelatin (Dernby, 1917). Such an experiment is repeated here, and Table III and Fig. 3 show the result.

Two optima are seen, one at pH = 3.5, corresponding to that of gastric pepsin, the other at pH = 7, corresponding to that of animal trypsin.

Liver.—Watery extracts obtained by grinding liver with sand were made in various manners, using fresh and half autolyzed liver, and extracts made with water, weak acids, and alkalies; but the extracts, which always contained much protein, attacked neither gelatin nor peptones. This was also true for spleen and confirms the statements of preceding authors; *e.g.*, Levene. On the other hand, if a

little of the liver suspension was mixed with gelatin, the gelatin was liquefied, and preliminary tests showed that this reaction took place only in acid solution. This reaction was first discovered by Theobald Smith (1894).

TABLE III.

Yeast Juice on Gelatin.

15 cc. gelatin + HCl or NaOH + water + 0.5 cc. yeast juice = 30 cc.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for			
	HCl	NaOH		1 hr.	3 hrs.	6 hrs.	24 hrs.
1	26.0 cc. 0.1 N	—	3.2	1	2½	4	4
2	16.0 " 0.1 "	—	3.5	1½	4	5	5
3	10.0 " 0.1 "	—	3.7	1½	3½	5	6
4	6.0 " 0.1 "	—	4.0	½	2	3½	6
5	—	—	4.5	0	2	3	6
6	—	1.9 cc. 0.1 N	5.1	0	3	6	6
7	—	3.0 " 0.1 "	5.8	1	5	6	6
8	—	4.0 " 0.1 "	6.7	1½	6	6	6
9	—	5.0 " 0.1 "	7.2	2	6	6	6
10	—	6.0 " 0.1 "	7.9	0	4	6	6

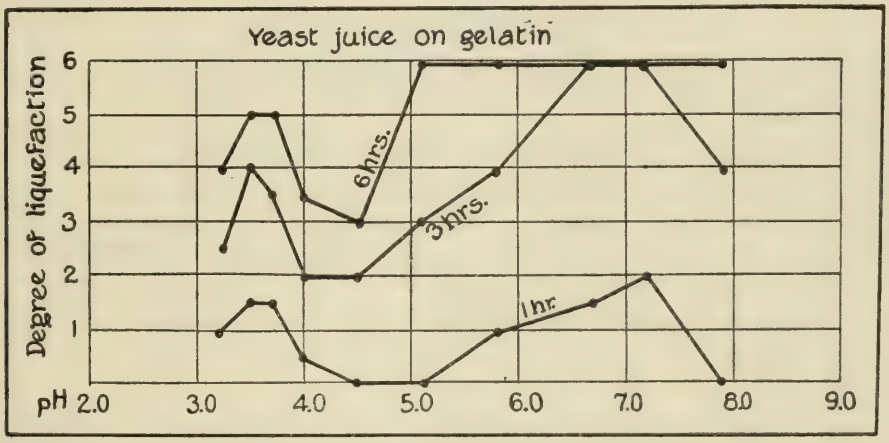


FIG. 3.

Tables IV and V give the results of two similar experiments. In Fig. 4 the results from Table V are graphically reproduced.

Tables IV and V show that the proteolytic action on gelatin takes place only in acid solution, and the optimum hydrogen ion concentration is between pH = 3 and pH = 4. Several similar experi-

TABLE IV.

Liver Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. liver suspension (fresh) + water = 30 cc.

1 cc. liver suspension contained 12.0 mg. total nitrogen.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for				
	HCl	NaOH		1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
1	4.0 cc. N	—	2.6	0	0	0	0	0
2	2.6 " "	—	3.0	0	0	0	1	2
3	1.6 " "	—	3.4	1	2	3	4	6
4	10.0 " 0.1 N	—	3.8	0	1	2	3	5
5	6.0 " 0.1 "	—	4.4	0	0	1	2	4
6	—	—	5.1	0	0	0	1	3
7	—	1.8 cc. 0.1 N	5.8	0	0	0	0	1
8	—	3.0 " 0.1 "	6.8	0	0	0	0	0
9	—	4.0 " 0.1 "	7.3	0	0	0	0	0
10	—	6.0 " 0.1 "	7.8	0	0	0	0	0

TABLE V.

Liver Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. liver suspension (4 days in ice box) + water = 30 cc.

1 cc. liver suspension contained 22.6 mg. total nitrogen.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for		
	HCl	NaOH		1 hr.	2 hrs.	3 hrs.
1	2.6 cc. N	—	3.2	0	2	3
2	1.6 " "	—	3.5	2	3½	5
3	10.0 " 0.1 N	—	3.7	3	3½	5
4	6.0 " 0.1 "	—	4.0	2	2½	4
5	—	—	4.5	1	1	3
6	—	1.8 cc. 0.1 N	5.1	0	1	2
7	—	3.0 " 0.1 "	5.8	0	0	0
8	—	4.0 " 0.1 "	6.7	0	0	0
9	—	5.0 " 0.1 "	7.2	0	0	0

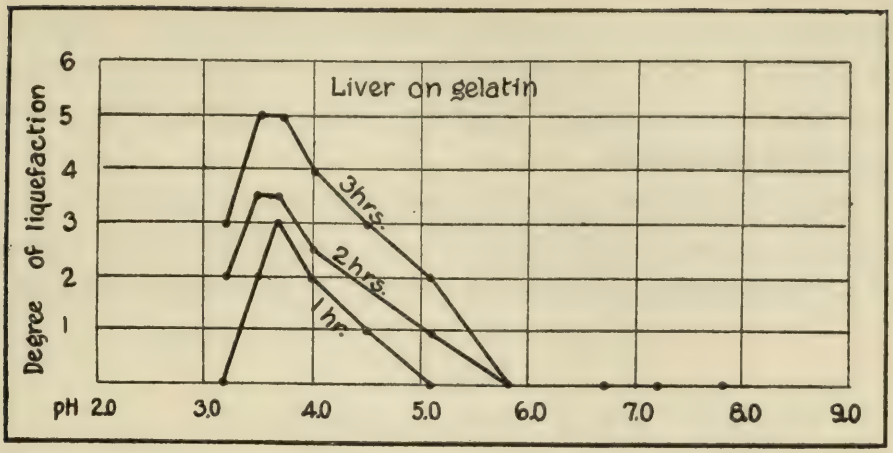


FIG. 4.

ments gave the same result, and it must especially be noted that in no case was any proteolytic action whatsoever obtained on the alkaline side. In this respect the liver and spleen, stomach cells, and leukocytes differ from pancreas and yeast cells.

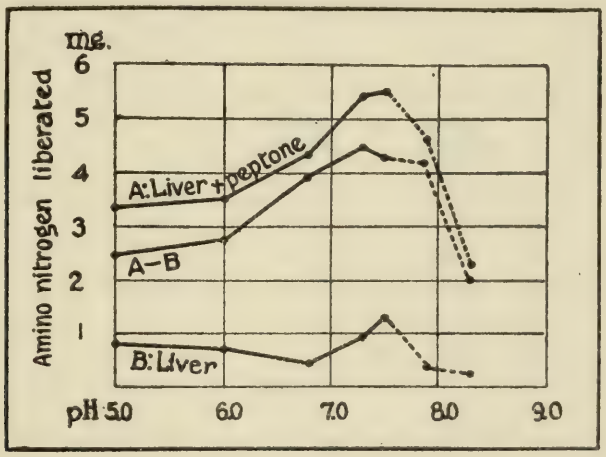


FIG. 5.

Tryptase.—Preliminary experiments showed that liver suspension readily attacked a solution of Witte's peptone, and that the rate of reaction was greatest in weakly alkaline solution. The objection may be raised that when peptone solution is mixed with such large amounts of liver suspension the amount of total nitrogen of the liver exceeds that of the peptone. One cannot tell whether the amino nitrogen formed originates from the peptone or from the liver tissue.

Table VI shows also a control experiment without any peptone, which indicates that comparatively small amounts of amino nitrogen are formed from the liver tissue alone. In Fig. 5 the results are represented graphically.

A considerable increase in the hydrogen ion concentration occurred during the digestions in spite of the presence of the phosphates. This was presumably due to the formation of acids from liver glyco-

TABLE VI.

Liver Suspension on Peptone.

A: 9 cc. peptone solution + 1 cc. liver suspension (7 days in ice box) + 1 cc. 0.5 M phosphates = 10 cc.

B: 1 cc. liver suspension + 1 cc. 0.5 M phosphates + HCl or NaOH + water = 10 cc.

1 cc. liver suspension contained 22.60 mg. total nitrogen.
1 " " " " 2.90 " amino "
9 " peptone solution " 15.84 " total "
9 " " " " 1.82 " amino "

Tube No.	pH			Increase in amino nitrogen after 48 hrs. at 37°.		
	Initial.	After digestion, A.	After digestion, B.	A.	B.	A − B (amino nitrogen formed from peptone).
				mg.	*mg.*	*mg.*
1	5.0	5.0	5.0	3.34	0.85	2.49
2	6.0	5.8	5.7	3.51	0.73	2.78
3	6.8	6.5	6.5	4.36	0.45	3.91
4	7.3	6.9	6.8	5.48	0.98	4.50
5	7.6	7.1	7.1	5.55	1.26	4.29
6	7.9	7.3	7.3	4.62	0.39	4.23
7	8.3	7.4	7.2	2.25	0.23	2.02

gen. Greater concentration of buffer salts to reduce the change did not appear advisable because of the effect of higher concentrations of salts in checking enzyme action.

The curve for liver digesting alone is noteworthy. At pH = 7.8 there is a marked maximum, at the neutral point there is very little reaction, but in the acid region there seems to be another maximum. This phenomenon will be further discussed under "Autolysis."

The main result obtained from this experiment is that for the digestion of peptone by liver enzymes the optimum pH is about 7.6.

Soluble Ereptase.—Abderhalden and collaborators (1906, 1907, 1916) and others have found that press juice from liver and other tissues has the power to split simple polypeptides into amino-acids. As already mentioned, watery extracts digest neither gelatin nor peptone, but Table VII shows an experiment in which the action of a liver extract on glycyl-glycine has been tried.

TABLE VII.

Liver Extract on Glycyl-Glycine.

5 cc. 0.1 M glycyl-glycine + 2 cc. 0.5 M phosphates + NaOH + 1 cc. liver extract + water = 20 cc.

5 cc. 0.1 M glycyl-glycine contained 14.0 mg. total nitrogen.

1 cc. liver extract " 1.28 " " "

Tube No.	pH		Increased amount of amino nitrogen after 20 hrs. at 37°.
	Initial.	After digestion.	
			mg.
1	5.4	5.4	0.05
2	6.8	6.6	0.90
3	7.1	6.8	1.16
4	7.4	7.1	1.40
5	7.7	7.4	1.62
6	8.0	7.6	1.78
7	8.4	7.7	3.03
8	9.0	7.9	0.0

A slight proteolytic action is evident, and the optimum lies on the alkaline side. It is, however, impossible to determine the optimum exactly because acid-forming substances in the liver cause a great change in the hydrogen ion concentration to the acid side.

Spleen.—Preliminary experiments with spleen suspensions indicated that the proteolytic activities of spleen are analogous to those of liver. Table VIII and Fig. 6 give the result of the gelatin test.

Tryptases.—Spleen tissue is more favorable to deal with than liver because the hydrogen ion concentration does not change so

TABLE VIII.

Spleen Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. spleen suspension (2 days in ice box) + water = 30 cc.

1 cc. spleen suspension contained 13.05 mg. total nitrogen.

Tube No.	Added.		pH		Degree of liquefaction after digestion at 37° for				
	HCl	NaOH	Initial.	After 6 hrs. digestion.	1 hr.	2 hrs.	3 hrs.	6 hrs.	20 hrs.
1	2.6 cc. N	—	3.2	3.4	½	1	2½	3	3½
2	1.6 " "	—	3.5	3.6	1	2	3	4	5
3	10.0 " 0.1 N	—	3.7	3.8	1	2	3½	4	5
4	6.0 " 0.1 "	—	4.0	4.1	½	1	2	2½	3
5	—	—	4.5	4.5	0	½	1	2	2
6	—	7.8 cc. 0.1 N	5.1	5.1	0	0	0	1	1
7	—	3.0 " 0.1 "	5.8	5.7	0	0	0	0	0
8	—	4.0 " 0.1 "	6.7	6.6	0	0	0	0	0
9	—	6.0 " 0.1 "	7.4	7.0	0	0	0	0	0

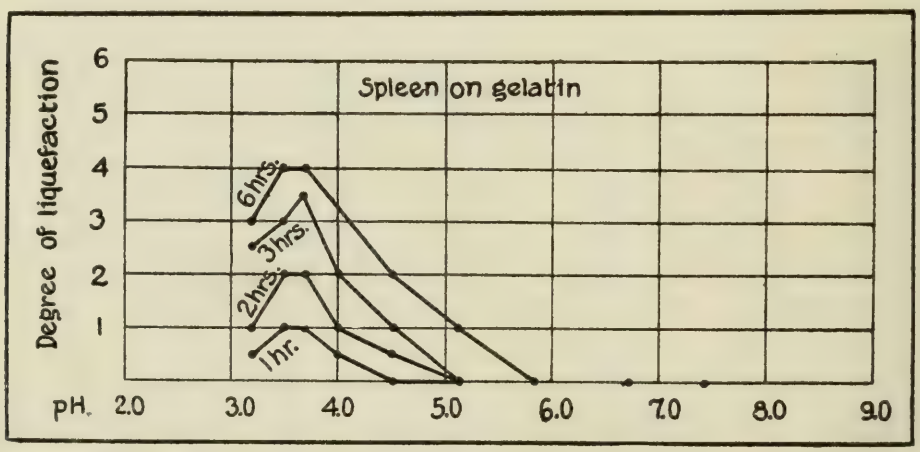


FIG. 6.

extensively in spleen. Table IX is quite similar to the corresponding Table VI for liver. Fig. 7 gives the results graphically.

Soluble Ereptase.—To see whether there is any ereptase present in a watery extract from spleen the following test was carried out. The extract was prepared in the same way as in the case of liver.

The proteolytic action is rather weak and far from a complete decomposition of the peptide, but it is quite evident that there are ereptases present in the extract, and the optimum for their action seems to be about pH = 8.

TABLE IX.

Spleen Suspension on Peptone.

9 cc. peptone solution + 1 cc. spleen suspension (fresh).
1 " spleen suspension contained 13.05 mg. total nitrogen.
1 " " " " 1.02 " amino "
9 " peptone solution " 15.84 " total "
9 " " " " 1.82 " amino "

Tube No.	pH		Liberated amino nitrogen after 18 hrs. at 37°.
	Initial.	After digestion.	
			mg.
1	6.0	5.9	1.96
2	6.8	6.6	1.92
3	7.3	7.1	2.47
4	7.6	7.4	2.92
5	7.9	7.6	2.64
6	8.3	8.0	0.39

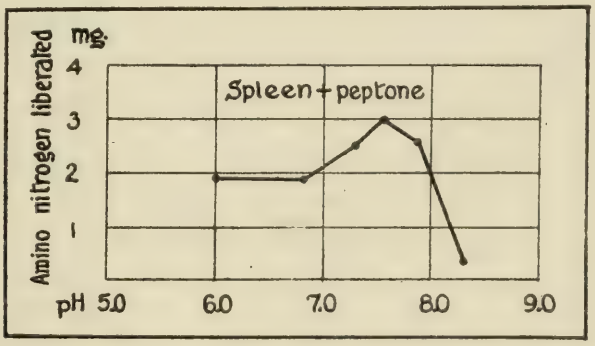


FIG. 7.

Pancreas.—The pancreas excretes large amounts of trypsin, which is readily obtained in watery extracts from the tissue. Palitzsch and Walbum have determined the optimum hydrogen ion concentration for the action of trypsin on gelatin, and found the astonishingly high value of pH = 9.1 at 35°. On the other hand Rona and Arnheim (1913) have found the optimum for the tryptic action on Witte's

peptone to be at pH = 7.8. Perhaps the liquefaction of gelatin in very alkaline solutions is due in part to other effects than that of the enzymes.

TABLE X.

Spleen Extract on Glycyl-Glycine.

5 cc. 0.1 M glycyl-glycine + 2 cc. 0.5 M phosphates + NaOH + 1 cc. spleen extract + water = 20 cc.

5 cc. 0.1 M glycyl-glycine contained 14.0 mg. total nitrogen.
1 cc. spleen extract " 0.88 " " "

Tube No.	pH		Increased amount of amino nitrogen after 18 hrs. at 37°.
	Initial.	After digestion.	
			mg.
1	5.4	5.4	0.00
2	6.8	6.8	0.28
3	7.1	7.1	0.57
4	7.4	7.4	0.69
5	7.7	7.6	0.80
6	8.0	7.8	0.94
7	8.4	8.1	0.57

TABLE XI.

Pancreas Extract on Gelatin.

15 cc. gelatin + HCl or NaOH + 3 cc. 0.5 M phosphates + 0.3 cc. pancreas extract + water = 30 cc.

Tube No.	Added.				Initial pH.	Degree of liquefaction after digestion at 37° for				
	HCl	NaOH	Phosphates.			15 min.	30 min.	60 min.	120 min.	20 hrs.
			Na_2HPO_4	KH_2PO_4						
			cc.	*cc.*						
1	9.0 cc. 0.1 N	—	0	3.0	4.1	0	0	1	2	6
2	—	—	0	3.0	5.0	0	1	3	4	6
3	—	1.0 cc. 0.1 N	0.6	2.4	6.0	0	3	4	5	6
4	—	3.0 " 0.1 "	1.5	1.5	6.8	2	4	5	6	6
5	—	4.0 " 0.1 "	2.4	0.6	7.3	3	5	6	6	6
6	—	6.0 " 0.1 "	2.7	0.3	7.8	4	5	6	6	6
7	—	8.0 " 0.1 "	3.0	0	8.2	4	5	6	6	6
8	—	1.4 " N	3.0	0	8.6	3	4	5	6	6
9	—	2.0 " "	3.0	0	9.3	3	4	4	5	6

In Table XI and Fig. 8 is presented an experiment which is a repetition of Palitzsch and Walbum's. Phosphates are used as buffers to keep the hydrogen ion concentration constant. It must be remembered that for measuring the hydrogen ion concentration instead of the electrometric method the colorimetric method with the indicators proposed by Clark has been used. It is a little difficult to locate exactly the optimum but it is evident that it is about or above pH = 8.

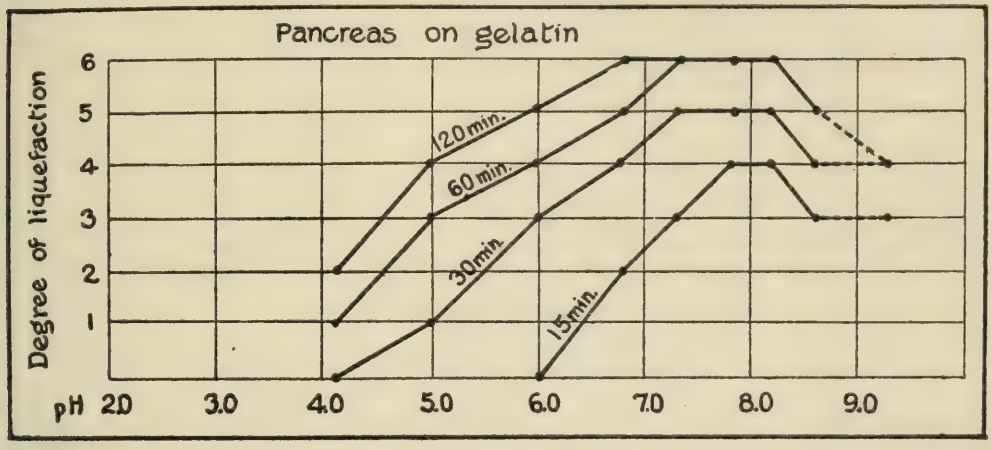


FIG. 8.

Repeated experiments, in which instead of pancreas extracts pancreas suspension was used, indicated that the latter had the power to liquefy gelatin within the limits pH = 3 to pH = 9. The optimum seemed to be in all cases about pH = 8. In the acid region liquefaction was very slow. Only one optimum, the alkaline one, was observed, and there was therefore no evidence of the existence of pepsin-like enzymes. Table XII shows one of these experiments.

In the pancreas there is so much trypsin that it may obscure the effects of other enzymes which may be present. The attempt was made therefore to get rid of most of the trypsin by washing the tissue mash thoroughly with distilled water, in order that the residue might be tested for other enzymes. Table XIII and Fig. 9 represent such an experiment.

Fig. 9 with its two optima is quite analogous to that representing the action of yeast juice on gelatin. It appears that the pancreas

TABLE XII.

Pancreas Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. pancreas suspension + water = 30 cc.

Tube No.	Initial pH.	Degree of liquefaction after digestion at 37° for			
		30 min.	1 hr.	2 hrs.	24 hrs.
1	3.0	0	0	2	6
2	3.3	0	0	4	6
3	3.7	0	1	5	6
4	4.0	0	3	6	6
5	4.5	1	5	6	6
6	5.1	2	6	6	6
7	5.8	4	6	6	6
8	6.8	5	6	6	6
9	7.5	6	6	6	6
10	7.8	6	6	6	6

TABLE XIII.

Pancreas Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. washed pancreas suspension + water = 30 cc.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for			
	HCl	NaOH		1 hr.	2 hrs.	3 hrs.	24 hrs.
1	2.6 cc. N	—	3.0	1	2	3	6
2	1.6 " "	—	3.4	2	3	4	6
3	10.0 " 0.1 N	—	3.8	2	2	3	6
4	6.0 " 0.1 "	—	4.4	0	0	1	6
5	—	—	5.1	0	0	1	6
6	—	1.8 cc. 0.1 N	5.8	0	1	3	6
7	—	3.0 " 0.1 "	6.8	1	2	4	6
8	—	4.0 " 0.1 "	7.3	2	3	5	6
9	—	6.0 " 0.1 "	7.8	2	4	5	6

contains not only trypsin but also pepsin, the trypsin so predominating that it must be partially removed before the pepsin can be detected.

Table XIV and Fig. 10 show the action of trypsin on peptone. Here we observe the effects of only the tryptic enzymes, and the experiment confirms the above mentioned observations by Rona and

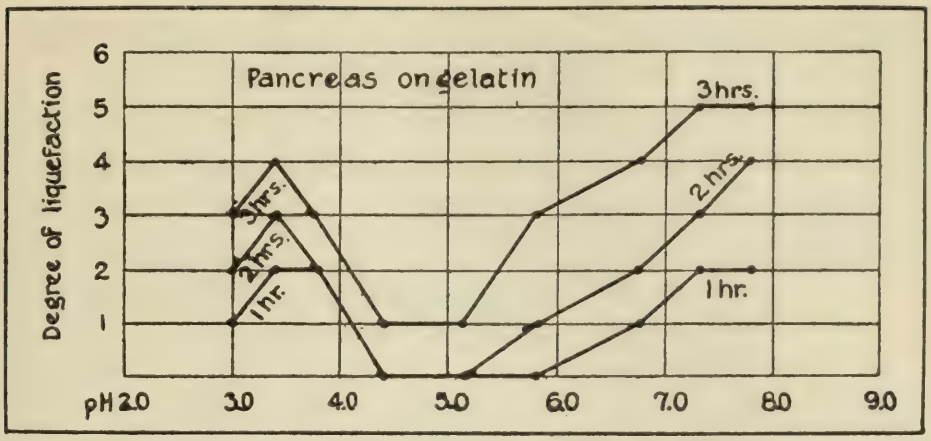


FIG. 9.

TABLE XIV.

Pancreas Extract on Peptone.

9 cc. peptone solution + 1 cc. pancreas extract = 10 cc.
9 " " " contained 15.84 mg. total nitrogen.
9 " " " " 1.82 " amino "
1 " pancreas extract " 3.40 " total "
1 " " " " 0.73 " amino "

Tube No.	Initial pH.	Amino nitrogen liberated after digestion at 37° for	
		2 hrs.	18 hrs.
		mg.	*mg.*
1	5.0	0.94	1.77
2	6.0	0.94	2.41
3	6.8	1.22	3.34
4	7.3	1.66	3.44
5	7.6	1.85	4.16
6	7.9	1.77	4.22
7	8.3	0.77	3.34

Arnheim (1913), that the optimum for the action of trypsin on peptone is at pH = 7.8.

Table XV shows the action of pancreas extract on glycyl-glycine, which is rather easily attacked. The optimum seems to be about pH = 7.9 and is also the same as for the action of erepsin from the intestines on glycyl-glycine. In this respect pancreas extract differs from yeast juice, because the optimum for its action on glycyl-glycine is at pH = 7.8 but the action on peptone is at about pH = 7.0.

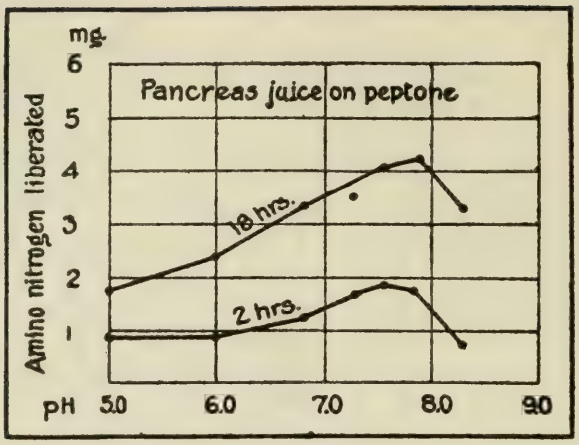


FIG. 10.

TABLE XV.

Pancreas Extract on Glycyl-Glycine.

5 cc. 0.1 M glycyl-glycine + 2 cc. 0.5 M phosphates + NaOH + 1 cc. pancreas extract + water = 20 cc.

5 cc. glycyl-glycine contained 14.0 mg. total nitrogen.

1 " pancreas extract " 1.10 " " "

Tube No.	pH		Increased amount of amino nitrogen after 18 hrs. at 37°.
	Initial.	After digestion.	
			mg.
1	5.4	5.4	0.40
2	6.8	6.8	1.60
3	7.1	7.1	2.14
4	7.4	7.5	2.56
5	7.7	7.9	3.02
6	8.0	8.1	2.62
7	8.4	8.4	2.54
8	>9.0	>9.0	0.00

Mucous Membrane of the Stomach.—The stomach of a freshly killed dog was cleaned by running water through, the ventricle was opened, and the mucous membrane was removed by means of a piece of glass. About 100 gm. were obtained. Toluene and chloroform were added. Part of the material was ground with sand in a mortar, and the mass afterwards extracted with distilled water. The insoluble parts were filtered off, and the yellowish turbid filtrate was used for the gelatin test.

To the other portion was added 0.5 gm. of powdered calcium carbonate in order to make the mixture neutral, and the mixture was allowed to stand for 24 hours at room temperature. The mash was washed several times by decantation with distilled water in order to remove most of the pepsin. Then the mash was diluted to twice the volume and shaken until a rather uniform suspension was obtained. This suspension was used for the peptone test.

Table XVI shows the result of the gelatin test.

TABLE XVI.

Stomach Extract on Gelatin.

15 cc. gelatin + HCl or NaOH + water + 0.5 cc. pepsin = 30 cc.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for			
	HCl	NaOH		15 min.	30 min.	60 min.	20 hrs.
1	10 cc. N	—	About 1.5	3	5	6	6
2	4 " "	—	2.5	5	6	6	6
3	8 " 0.1 N	—	3.0	4	6	6	6
4	3 " 0.1 "	—	4.0	2	4	5	6
5	—	—	5.1	1	2	3	5
6	—	1.8 cc. 0.1 N	6.5	0	0	1	1
7	—	3.0 " 0.1 "	7.3	0	0	0	0
8	—	4.0 " 0.1 "	7.8	0	0	0	0
9	—	6.0 " 0.1 "	8.5	0	0	0	0

It is obvious that the water extract from the stomach contains large amounts of pepsin. The results are in marked contrast to those obtained with water extracts from liver, pancreas, and spleen, which showed no trace of peptic enzymes. The optimum for the action is in the acid region and seems to lie about pH = 2 to 3. It is impossible to locate the optimum very accurately by this method, because in order to obtain hydrogen ion concentrations greater than pH = 2, such large amounts of acid must be added that they interfere with the liquefaction of the gelatin. The gelatin method as has been said is only useful for values of pH between 2 and 8.

Preceding authors have made accurate determinations of the optimum hydrogen ion concentration for pepsin, and found it to lie between a pH of 1 and 3. Sörensen (1909) found for the digestion of

acid albumin an optimum pH of 1.5 which later was confirmed by Okada (1916). Recently Ringer (1916) has published a very interesting paper, showing that the optimum varies with the concentration and nature of the ions present. It is evident, however, from this experiment that there is no proteolytic action at all on gelatin above a pH of 6.

The question remains as to whether the mucous membrane of the stomach contains trypsin. It is not likely to be found in the extract, because if it entered the acid solutions in the stomach it probably would be destroyed, as pancreatic trypsin is under similar conditions. For the peptone test, therefore, the mash has been used, the preparation of which is described above. Table XVII shows the results of this experiment.

TABLE XVII.

Stomach Cells on Peptone.

9 cc. peptone mixture + 1 cc. tissue suspension = 10 cc.
9 " " " contained 15.84 mg. total nitrogen.
1 " tissue suspension " 6.20 " " "

Tube No.	Initial pH.	Increase in the liberated nitrogen.
		mg.
1	5.0	<1.0
2	6.0	2.75
3	6.8	3.40
4	7.3	3.63
5	7.6	4.12
6	7.9	3.82
7	8.3	3.46

From this table it is quite evident that there are enzymes present which have the power of digesting Witte's peptone. The well marked optimum appears to lie at about pH = 7.6, about the same as for tryptic enzymes in the other tissues examined. That the pepsin itself cannot digest peptone in acid solution is shown in the following experiment.

2 cc. 4 per cent Witte's peptone solution + 4 cc. N HCl + 1 cc. pepsin solution + water = 10 cc.
At the beginning were obtained..... 0.91 mg. N_2.
After 24 hours digestion at 37°..... 0.90 " N_2.

These experiments have shown that there are in the ventricle, besides the well known pepsin, enzymes of the trypsin type, their presence however being largely obscured by the predominant action of pepsin.

Leukocytes.—Opie and later Dochez have studied the autolysis of polynuclear leukocytes and from their experiments concluded that proteolytic enzyme of leukocytes is active in alkaline solution. Parker and Franke (1917) claim to have demonstrated ereptic enzymes in rabbit leukocytes, which attack peptones but not proteins in alkaline solution. Other authors who have studied this problem are Jobling, Eggstein, and Petersen (1915), Fiessinger and Clogne (1917), and Hiss (1908).

The leukocytes used in the following experiment were obtained by injecting sterile aleuronat into the pleural cavity of a rabbit. The exudate was centrifugalized, and the cells were carefully washed with salt solution. The deposit proved under the microscope to consist almost entirely of polynuclear cells, though a few mononuclear cells could be seen. About 0.5 gm. wet weight was obtained. To it was added chloroform, and it was allowed to stand for 24 hours at about 20° in order to plasmolyze. Then the cells were suspended in 25 cc. of distilled water, and this suspension was used as enzyme preparation. Table XVIII and Fig. 11 show the result of this experiment.

TABLE XVIII.

Leukocytes on Gelatin.

15 cc. gelatin + HCl or NaOH + water + 0.2 cc. leukocyte suspension = 30 cc.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for			
	HCl	NaOH		1 hr.	2 hrs.	6 hrs.	24 hrs.
1	6.0 cc. N	—	About 2.0	1	2	4	5
2	2.0 " "	—	2.7	2	4	6	6
3	10.0 " 0.1 N	—	3.2	2	4	5½	6
4	7.0 " 0.1 "	—	3.7	1½	3½	5	6
5	9.0 " 0.1 "	—	4.3	1	2	3	6
6	—	—	4.9	0	1	2	4
7	—	1.2 cc. 0.1 N	6.0	0	0	1	2
8	—	1.8 " 0.1 "	6.8	0	0	0	1
9	—	2.4 " 0.1 "	7.3	0	0	1	1
10	—	3.2 " 0.1 "	7.7	0	0	1	1
11	—	5.0 " 0.1 "	8.3	0	0	1	1

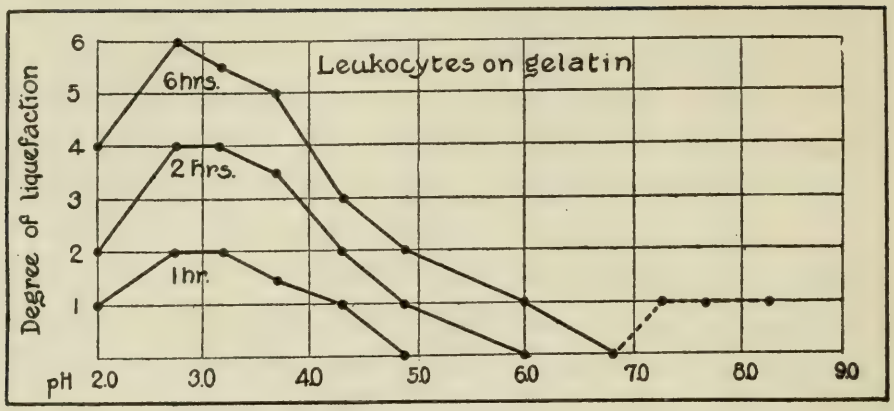


FIG. 11.

The gelatin test indicates that leukocytes, like the other tissues examined, contain pepsin-like enzymes with maximum activity at pH = 3. There is no evidence of any proteolytic enzyme acting in alkaline solution on gelatin. Repeated experiments gave the same result.

The fact is noteworthy that the small quantity of leukocytes used could digest gelatin so rapidly. In the case of spleen or liver we had to use several hundred times as much tissue in order to get a corresponding action. The leukocytes evidently contain much greater amounts of pepsin-like enzymes than the other tissues. In this respect they resemble the yeast cells more than liver cells.

Table XIX shows the action of leukocytes on Witte's peptone.

TABLE XIX.

Leukocytes on Peptone.

9.0 cc. peptone mixture + 0.3 cc. leukocyte suspension + water = 10 cc.
Total nitrogen = 15.84 mg.
Amino " = 1.82 "

Tube No.	Initial pH.	Increase in the liberated amino nitrogen after 48 hrs. at 37°.
		mg.
1	4.0	0.17
2	5.3	0.17
3	6.8	0.25
4	7.3	0.85
5	7.6	1.02
6	7.9	1.68
7	8.5	0.25

Even if the resulting differences are small it is evident that there must also be trypsin-like enzymes present in the leukocytes, and their optimum reaction seems to be the same as that for the other animal tissues, between pH = 7.6 and pH = 8.0.

Autolysis.

The preceding experiments have yielded information concerning the nature of the proteolytic enzymes found in the tissues, and we have used a similar technique to study the nature of autolysis itself.

Liver.—Presumably because the glycogen present forms organic acids during autolysis, it is difficult to keep the hydrogen ion concentrations of liver mixtures constant during incubation. The following experiments show how strongly the hydrogen ion concentration increases.

Liver, autolyzed for 72 hours. No buffer present.

Tube No.	Initial pH.	pH after digestion.
1	6.1	5.5
2	7.3	6.4
3	7.9	6.8
4	8.4	6.9

Therefore we must use buffers and choose a rather short digestion time. In Table VI it is shown that the amino nitrogen formation at almost constant hydrogen ion concentration follows a curve of somewhat peculiar shape. We have two optimal points, one in the acid, the other in the alkaline region, and at about the neutral point there is almost no action at all. Table XX and Fig. 12 show a similar, more elaborate experiment.

In spite of the phosphates present the hydrogen ion concentration increased appreciably, especially after 72 hours digestion. For this reason only the results after 20 hours digestion are used for plotting the curve. We note two well defined maxima in the curve, one at a pH of 7.8, the other at a pH of 5.0. There is greater action in the acid region, especially after longer duration of time. The optimum at pH = 7.8 is probably that of the tryptase which attacks the pep-

TABLE XX.

Autolysis of Liver.

1 cc. liver suspension (kept 3 days in ice box) + HCl or NaOH + 1 cc. 0.5 M phosphates + water = 10 cc.

Total nitrogen per 10 cc. = 22.60 mg.

Amino " " 10 " = 2.57 "

Tube No.	Added.				Initial pH.	After 20 hrs. digestion at 37°.		After 72 hrs. digestion at 37°.	
	0.1 N HCl.	0.1 N NaOH.	0.5 M phosphates.			Increase in amino N.	pH after digestion.	Increase in amino N.	pH after digestion.
			Na_2HPO_4	KH_2PO_4					
	cc.	*cc.*	*cc.*	*cc.*		*mg.*		*mg.*	
1	6.0	—	—	—	3.1	0.69	3.2	1.43	3.5
2	2.5	—	—	—	4.0	2.52	4.2	3.57	4.4
3	1.0	—	0	1.0	5.0	3.86	5.1	4.57	5.1
4	—	—	0.2	0.8	6.1	2.35	6.1	4.14	5.5
5		0.1	0.5	0.5	6.8	1.43	6.7	3.14	5.9
6		0.25	0.8	0.2	7.4	1.75	7.2	1.89	6.4
7		0.5	0.9	0.1	7.9	1.86	7.4	2.23	6.5
8		1.3	1.0	0	8.4	0.23	7.6	0.63	6.8

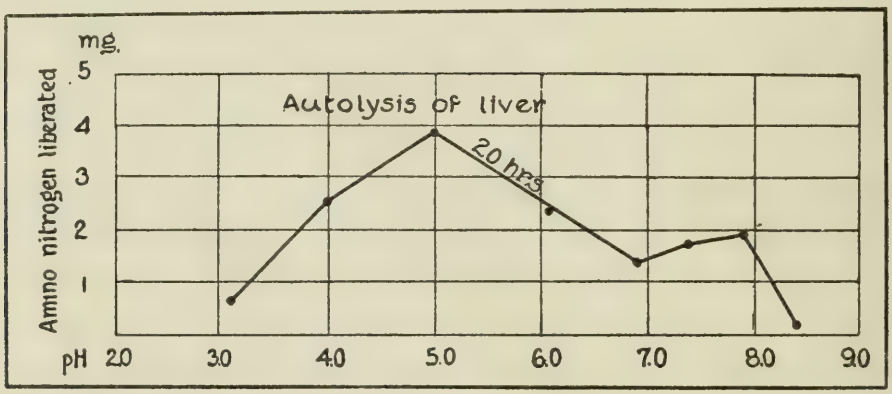


FIG. 12.

tones already present. But what does the other maximum mean? Do we have another enzyme of trypsin type, acting in slightly acid solution? In such case we ought to have detected it before. Experiments in Tables IV to VI give no enzyme having the optimum pH = 5.0. But bearing in mind that the formation of amino nitrogen results from the action of both enzymes—the trypsin hydrolyzing the albumoses and peptones, first formed by the pepsin—it is easy to see

that the optimum pH for the total action may well lie between the optima of the two individual enzymes and at a point where each exerts only a part of its maximum activity. The observed optimum at a pH of 5.0 occupies such a position.

Since pepsin produces only the more complex proteolytic products, such as albumoses and peptones, while trypsin produces amino-acids and simple peptides, it is of interest to study the complexity of the products formed by autolysis at different hydrogen ion concentrations. When working with yeast it was found that in acid solutions considerable amounts of non-precipitable peptide nitrogen were present, whereas after autolysis in alkaline solutions practically all of the non-protein nitrogen was converted into amino nitrogen. A previous test gave the following result.

TABLE XXI.

Autolysis of Liver Products at Different Hydrogen Ion Concentrations.

25 cc. fresh pig liver suspension + HCl or NaOH + water = 100 cc.
Total nitrogen = 229.0 mg.
Amino " = 15.0 "

Tube No.	Added.		pH		Nitrogen in filtrate after 72 hrs. at 37°.			
	0.1 N HCl.	0.1 N NaOH.	Initial.	After digestion.	Total.	Protein.	Peptide.	Amino.
	cc.	*cc.*			*mg.*	*mg.*	*mg.*	*mg.*
1	20	—	4.2	4.0	177.0	9.0	63.0	64.5
2	—	10	7.6	7.0	132.0	24.0	0.0	51.8

As stated before it is impossible to keep the hydrogen ion concentration constant when working with liver, but when this organ is used it is determined before and after each digestion. Table XXII shows a similar experiment in which digestion was stopped by adding trichloroacetic acid. The coagulum was removed and the amounts of peptide, amino, and ammonia nitrogen were determined as described under "General methods."

It is evident from the table that when autolysis has proceeded in acid solution a large proportion of peptide nitrogen still is left; that is, almost all of the dissolved proteins have been split to intermediate products, albumoses and peptones. But after autolysis in alkaline

TABLE XXII.

Autolysis Products at Different Hydrogen Ion Concentrations.

25 cc. liver suspension (kept on ice for 7 days) + HCl or NaOH + water = 100 cc.

Total nitrogen = 402.0 mg.
Peptide " = 7.5 "
Amino " = 47.0 "
NH_3 " = 3.3 "

Tube No.	Added.	pH		Amount of non-preciptable nitrogen in filtrate after 48 hrs. at 37°.		
		Initial.	After digestion.	Peptide.	Amino.	NH_3
				mg.	*mg.*	*mg.*
1	12.0 cc. N HCl.	2.8	3.1	33.2	57.3	3.3
2	3.0 " " HCl.	4.4	3.9	37.7	118.3	6.2
3	—	6.2	5.5	12.2	95.0	9.5
4	1.5 cc. N NaOH.	7.9	7.2	3.9	64.1	5.6

solution we have a large amount of intact soluble proteins and amino-acids, with no intermediate products.

Bearing in mind that the pepsin of the tissues is active only in acid, and the tryptase only in alkaline solution, the results described may readily be explained without any assumption of hypothetical activators or catalysts, or pro- or antienzymes.

Effect of Pre-Treatment with Acids.—Preceding authors have paid much attention to the phenomenon that a piece of tissue does not autolyze in alkaline solution, but if pre-treated with acid and then transferred into alkaline solution it will undergo a rapid autolysis. In the light of the results reported above the following explanation of the fact seems probable. In alkaline solutions the tissue pepsin does not attack most of the proteins, and the trypsin can digest only the peptones which are present from the beginning. But during the pre-treatment with acid the pepsin becomes active and the proteins are partially converted into peptones, which in the alkaline solution later proceed to undergo further digestion by the tissue trypsin. Without the pre-treatment in acid most of the material would have remained in the form of native protein indigestible by trypsin.

Here are given the results of an experiment which was carried out in the following manner. 3 equal parts of mashed liver were put into Erlenmeyer flasks, to two of them, a_1 and a_2, were added the same amounts of hydrochloric acid, and to the third, b, was added sodium hydroxide. The amount of amino nitrogen in them was determined at the beginning. After a certain time so much sodium hydroxide was added to a_2 that the hydrogen ion concentration became the same as in b. To the other two were added the same volume of water, and all were allowed to autolyze for the same time. Then the increase in amino nitrogen was determined.

TABLE XXIII.

Influence of Pre-Treatment with Acids.

a_1 and a_2: 5 cc. liver suspension (7 days in ice box) + 5 cc. 0.1 N HCl + water = 25 cc.

b: 5 cc. liver suspension + 18 cc. 0.1 N NaOH + water = 25 cc.

Total nitrogen = 4.30 mg.

Amino " = 4.70 "

	a_1		a_2		b	
	Increase in amino N.	pH	Increase in amino N.	pH	Increase in amino N.	pH
	mg.		*mg.*		*mg.*	
At the beginning	—	4.7	—	4.7	—	7.8
After 20 hrs. digestion at 24°	1.79	4.5	1.79	4.5, transferred to 7.8	1.34	7.5
" 24 " additional digestion at 37°	3.55	4.2	2.79	7.3	1.46	7.1

Spleen.—Spleen is a more favorable tissue for studies of autolysis than liver, because it does not contain so much glycogen, the decomposition of which raises the hydrogen ion concentration during the digestion.

Experiments detailed on previous pages have shown that the liberation of amino nitrogen by autolysis at constant hydrogen ion concentration follows the same scheme as for liver. Table XXIV and Fig. 13 show the results.

TABLE XXIV.

Autolysis of Spleen.

1 cc. spleen suspension (fresh) + HCl or NaOH + 1 cc. 0.5 M phosphates + water = 10 cc.

Total nitrogen = 6.8 mg.
Amino " = 0.53 "

Tube No.	Added.				Initial pH.*	Increase in amino-acid per 10 cc. at 37°.	
	0.1 N HCl.	0.1 N NaOH.	Phosphates.			After 16 hrs.	After 72 hrs.
			Na_2HPO_4	KH_2PO_4			
	cc.	*cc.*	*cc.*	*cc.*		*mg.*	*mg.*
1	6.0	—	—	—	About 3.0	0.03	0.06
2	2.5	—	—	—	" 4.0	0.40	0.78
3	1.0	—	0	1.0	5.0	1.06	1.28
4	—	—	0.2	0.8	6.2	0.43	0.73
5	—	—	0.5	0.5	6.8	0.15	0.43
6	—	0.1	0.8	0.2	7.3	0.38	0.69
7	—	0.2	0.9	0.1	7.6	0.55	0.78
8	—	0.4	1.0	0	7.9	0.74	0.98
9	—	1.0	1.0	0	8.4	0.09	

* In this case the hydrogen ion concentration did not change so much as in the case of liver. On the acid side the hydrogen ion concentration remained rather constant; on the alkaline, however, it decreased 0.2 to 0.3 in pH during the digestion.

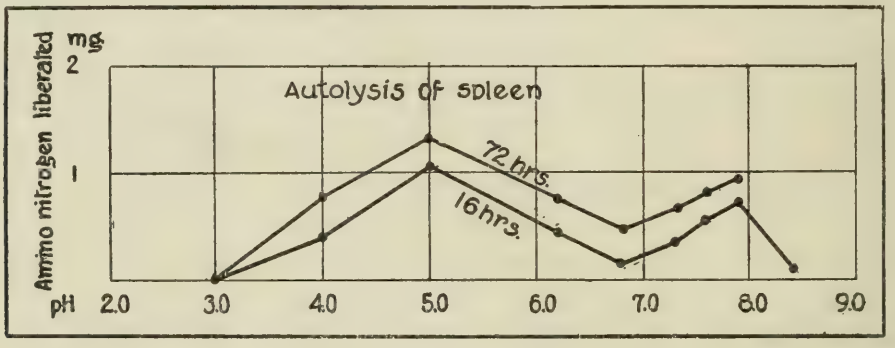


FIG. 13.

We have here two optima, one at a pH of about 8, the other at a pH of about 5. The explanation is the same as for the corresponding experiment with liver.

Table XXV shows the autolysis products at different hydrogen ion concentrations. It is analogous to Table XXII.

TABLE XXV.

Autolysis of Spleen and Products at Different Hydrogen Ion Concentrations.

25 cc. spleen suspension (quite fresh) + HCl or NaOH + water = 100 cc.
100 cc. contained 327.5 mg. total nitrogen.
100 " " 17.7 " peptide "
100 " " 25.5 " amino "
100 " " 2.3 " NH_3 "

Tube No.	Added.	pH		Amount of non-precipitable nitrogen after 48 hrs. at 37°.		
		Initial.	After digestion.	Peptide.	Amino.	NH_3
				mg.	*mg.*	*mg.*
1	9.0 cc. 0.1 N HCl.	2.9	3.1	24.8	38.5	2.3
2	1.0 " 0.1 " HCl.	4.2	3.9	55.2	142.3	6.2
3	—	6.3	5.9	2.6	87.9	7.3
4	1.0 cc. 0.1 N NaOH.	7.6	7.3	3.3	83.0	5.1

The most interesting results are to be found under "Peptide nitrogen." When autolysis has taken place in acid solutions, there are considerable amounts of intact peptides, whereas in alkaline solutions almost all peptides are split into amino-acids. This is the same result as obtained with liver, and subject to the same explanation.

Tables XXVI and XXVII show the influence of pre-treatment with acids and subsequent transfer to alkaline solution. In both cases autolysis proceeds much more rapidly in the transferred sample than in the one where the reaction is alkaline from the beginning. Here also the results are the same as with liver.

Pancreas.—Most authors have found that pancreas tissue autolyzes much more rapidly than liver and spleen. This is undoubtedly due to the great amounts of free trypsin present. Yet the pancreas is not a good subject for studying the general problem of autolysis because the action of the trypsin almost entirely obscures the actions of the other proteolytic enzymes. This has been shown in Table XI.

Table XXVIII is quite similar to Tables XX and XXIV, and shows the liberation of amino nitrogen during autolysis at constant hydrogen ion concentration. Fig. 14 represents the results graphically.

In the case of the liver and spleen we obtained two maximum points, one in the acid and one in the alkaline region. But here we

TABLE XXVI.

Influence of Pre-Treatment with Acids.

a_1 and a_2: 5 cc. spleen suspension (6 days in ice box) + 4.0 cc. 0.1 N HCl + water = 20 cc.

b: 5 cc. spleen suspension + 1.0 cc. 0.1 N NaOH + water = 20 cc.

a_2 neutralized after 16 hrs. with 5.5 cc. 0.1 N NaOH.

Total nitrogen = 34.0 mg.

Amino " = 2.68 "

	a_1		a_2		b	
	Increase in amino N.	pH	Increase in amino N.	pH	Increase in amino N.	pH
	mg.		*mg.*		*mg.*	
At the beginning	—	4.0	—	4.0	—	7.8
After 16 hrs. digestion at 24°	5.05	4.0	5.05	4.0, transferred to 7.8	1.20	7.8
" 18 " " " 37°	7.25	4.0	7.91	7.6	3.52	7.6
" 72 " " " 37°	8.10	4.2	9.36	7.6	3.94	7.6

TABLE XXVII.

a_1 and a_2: 5 cc. spleen suspension (fresh) + 2 cc. 0.1 N HCl + water = 20 cc.

b: 5 cc. spleen suspension + 0.8 cc. 0.1 N NaOH + water = 20 cc.

a_2 neutralized after 6 hrs. at 37° with 2.8 cc. 0.1 N NaOH.

Total nitrogen = 16.0 mg.

Amino " = 1.52 "

	a_1		a_2		b	
	Increase in amino N.	pH	Increase in amino N.	pH	Increase in amino N.	pH
	mg.		*mg.*		*mg.*	
At the beginning	—	4.7	—	4.7	—	7.6
After 6 hrs. digestion at 37°	0.31	4.7	0.31	4.7, transferred to 7.6	0.12	7.6
" 24 " " " 37°	2.58	4.6	1.80	7.6	0.65	7.5

TABLE XXVIII.

Autolysis of Pancreas.

1 cc. pancreas suspension (fresh) + HCl or NaOH + 1 cc. 0.5 M phosphates + water = 10 cc.

10 cc. contained 17.4 mg. total nitrogen.

10 " " 1.26 " amino "

Tube No.	Added.				Initial pH.	Increase in amino-acids per 10 cc. after digestion at 37° for	
	0.1 N HCl.	0.1 N NaOH.	Phosphates.			20 hrs.	72 hrs.
			Na_2HPO_4	KH_2PO_4			
	cc.	*cc.*	*cc.*	*cc.*		*mg.*	*mg.*
1	6.0	—	—	—	About 3.0	—	1.37
2	2.5	—	—	—	" 4.0	0.23	3.17
3	1.0	—	0	1.0	5.0	2.40	5.12
4	—	—	0.2	0.8	6.2	3.59	6.08
5	—	—	0.5	0.5	6.8	4.00	6.70
6	—	0.1	0.8	0.2	7.3	4.27	7.12
7	—	0.2	0.9	0.1	7.6	4.12	—
8	—	0.5	1.0	0	7.9	4.00	7.19
9	—	1.2	1.0	0	8.4	1.31	3.27

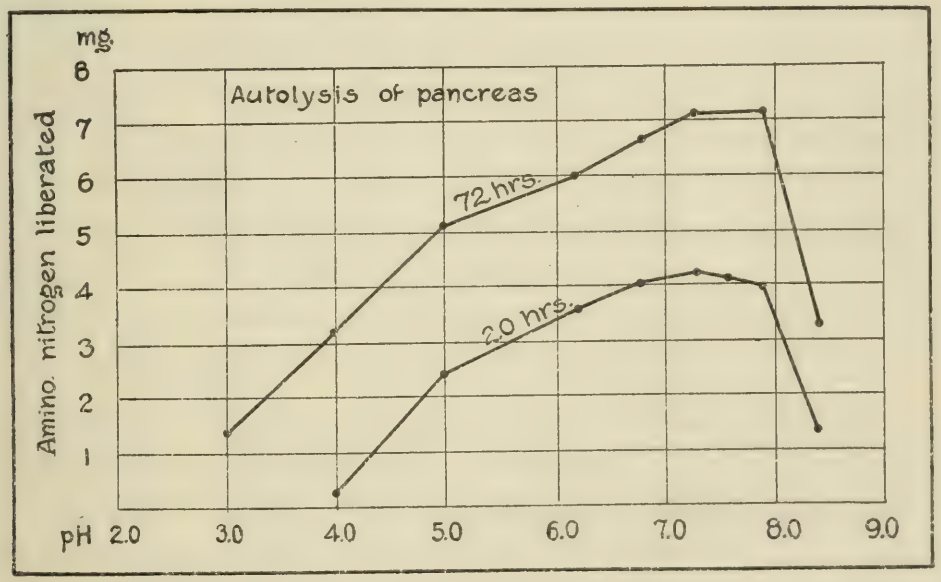


FIG. 14.

only obtain one maximum point, at about pH = 7.8, which also is the maximum for trypsin. Even in the acid region, however, there is a considerable proteolytic activity. Whether this is due to the trypsin or to the pepsin or to both is impossible to state.

Because of this irregular behavior of the pancreas it will hardly be of value to find out the autolytic products at different hydrogen ion concentrations. Table XXIX shows the influence of pre-treatment with acids on the subsequent autolysis in alkaline solution. It is quite similar to the corresponding experiments on liver and spleen, and gives the same result.

TABLE XXIX.

Influence of Pre-Treatment with Acids.

a_1 and a_2: 5 cc. pancreas suspension (7 days in ice box) + 3 cc. 0.1 N HCl + water = 20 cc.

b: 5 cc. pancreas suspension + 1 cc. 0.1 N NaOH + water = 20 cc.

After 6 hrs. digestion at 37° a_2 neutralized with 3.6 cc. 0.1 N NaOH.

Total nitrogen in 20 cc. = 63.1 mg.

Amino " " 20 " = 10.2 "

	a_1		a_2		b	
	Increase in amino N.	pH	Increase in amino N.	pH	Increase in amino N.	pH
	mg.		*mg.*		*mg.*	
At the beginning	—	4.8	—	4.8	—	7.8
After 6 hrs. digestion at 37°	2.70	4.9	2.70	4.9, transferred to 7.8	1.75	7.8
After subsequent 24 hrs. digestion at 37°	9.60	5.0	10.80	7.7	7.80	7.8

Influence of Neutral Salts on Animal Enzymes.

The erepsin of the intestines behaves in regard to neutral salts quite differently from ereptase of yeast. The ereptic action is markedly checked already in 0.1 normal salt solution, while the yeast ereptase is not influenced in less than about normal (Dernby, 1917). Whether this sensitiveness to salt concentration is shared by the other animal enzymes remains to be determined. It is likely that most of the enzymes from animal tissue are as sensitive to salts as erepsin.

SUMMARY.

In this paper the proteolytic enzymes of liver, spleen, pancreas, and leukocytes, and of the mucous membrane of the stomach have been studied. In all of these tissues it has been possible to demonstrate the existence of pepsin-like enzymes, which split native proteins to peptones but not further, and for whose action the optimum hydrogen ion concentration in all cases is at about pH = 3.5. This enzyme cannot act in alkaline or even neutral solutions.

It has also been possible to show that in all of these tissues there are proteolytic enzymes of the type of trypsin or erepsin that attack only peptones or peptides, and split them into amino-acids. The optimum hydrogen ion concentration for all these enzymes seems to be about pH = 7.8 (from 7.6 to 8.0), and their action is checked in slightly acid solution.

Both types of proteolytic enzymes occur in all the tissues studied. In some of them, however, one or the other of the enzymes has been predominant over the others, as the pepsin in the stomach and the trypsin in the pancreas. Unlike the less specialized enzymes, pancreatic trypsin and gastric pepsin can easily be extracted from the cells.

Autolysis of pancreas and liver has been especially studied in regard to the hydrogen ion concentration of the solution. It has been found that self-decomposition of the tissues proceeds furthest in a range of pH between 5 and 6; that is, in slightly acid solution and the optimum lies between those of pepsin and trypsin. The explanation is that the autolysis is most rapid and complete when both types of enzymes can work simultaneously.

The relations between hydrogen ion concentration and the different proteolytic enzymes as well as the autolysis of animal tissues are represented in Fig. 15.

When a piece of tissue is first treated with weak acids and subsequently transferred into alkaline solution the autolysis here proceeds much farther than if the tissue has been from the start in alkaline solution. This fact, shown previously by Hedin and by Dochez, is explained as follows. During the preliminary treatment in acid solution the pepsin acts. By transfer to alkaline solution its action is

checked, and the trypsin works under optimum conditions and digests the peptones formed by the action of the pepsin.

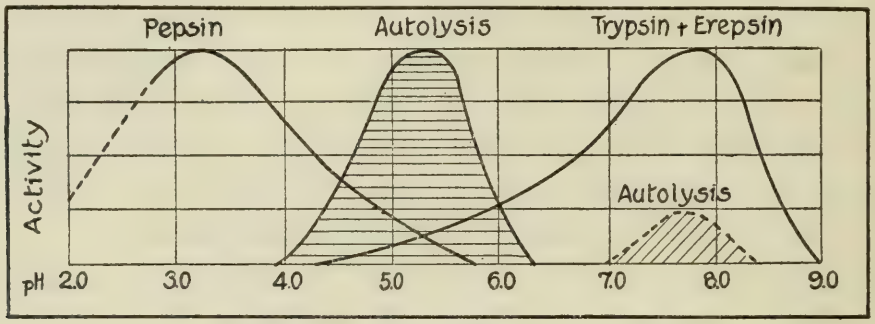


FIG. 15.

Considering that there are enzymes in the tissues of both peptic and tryptic nature and considering also the hydrogen ion concentration of the medium and its eventual changes, most of the autolytic reactions can be explained without assumption of hypothetical agents, such as "proenzymes," "activators," or "antienzymes."

Comparing the proteolytic actions in the yeast cells with those of animal tissues, the following is obtained:

Proteolytic Enzymes.	*Optimum Hydrogen Ion Concentration for the Enzymes.*	
	Yeast cell. *pH*	Animal tissues. *pH*
Pepsin	4.0–4.5	3.0–3.5
Trypsin	About 7	7.8
Erepsin	7.8	7.8

In what sense the hydrogen ion concentration will change during autolysis is impossible to predict. This depends upon the chemical constituents of different tissues and might vary in any direction. When starting at about pH = 6 in buffer-free solution with a glycogen-free tissue the most common result is that the hydrogen ion concentration first increases, reaches a maximum, and then decreases considerably.

This work has been carried out in the laboratory of Dr. Donald D. Van Slyke, and I wish to express my great indebtedness to him for his interest in this work. I am also indebted to Dr. J. Auer for supplying me with the animal tissues.

BIBLIOGRAPHY.

Abderhalden, E., and Fodor, A., *Fermentforsch.*, 1916, i, 533.
Abderhalden, E., and Koelker, A. K., *Z. physiol. Chem.*, 1907, li, 294.
Abderhalden, E., and Teruuchi, Y., *Z. physiol. Chem.*, 1906, xlvii, 466; xlix, 1.
Avery, O. T., and Dernby, K. G., *J. Exp. Med.*, 1918, xxviii, 345.
Baer, J., and Loeb, A., *Arch. exp. Path. u. Pharmakol.*, 1905, liii, 1.
Biondi, C., *Virchows Arch. path. Anat.*, 1896, cxliv, 373.
Bradley, H. C., *J. Biol. Chem.*, 1915, xx, p. xxix; 1916, xxv, 201.
Bradley, H. C., and Morse, M., *J. Biol. Chem.*, 1915, xxi, 209; xxii, 113.
Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, 1916, xxv, 261, 363.
Buchner, E., and Hahn, H., *Biochem. Z.*, 1909, xix, 191; 1910, xxvi, 171.
Buchner, M., and Hahn, H., Die Zymasegärung, Munich, 1903.
Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 109, 191.
Dernby, K. G., *Biochem. Z.*, 1917, lxxxi, 109.
Dochez, A. R., *J. Exp. Med.*, 1910, xii, 666.
von Euler, H., General chemistry of the enzymes, London, 1912.
von Euler, H., and Lindner, P., Chemie der Hefe und der alkoholischen Gärung, Leipsic, 1915.
Fermi, C., *Arch. Hyg.*, 1891, xii, 242; 1906, lv, 142.
Fiessinger, N., and Clogne, R., *Ann. méd.*, 1917, iv, 445.
Hedin, S. G., *J. Physiol.*, 1903–04, xxx, 155.
Hedin, Festskr. Olof Hammarsten, *Upsala Läkaref. Förh.*, Suppl., 1906, xi.
Hedin, S. G., and Rowland, S., *Z. physiol. Chem.*, 1901, xxxii, 531.
Hiss, P. H., *J. Med. Research*, 1908, xix, 320.
Jacobi, Y., *Z. physiol. Chem.*, 1900, xxx, 149.
Jobling, J. W., Eggstein, A. A., and Petersen, W., *J. Exp. Med.*, 1915, xxi, 239.
Landsteiner, K., *Centr. Bakteriol., Ite Abt.*, 1900, xxvii, 357.
Levene, P. A., *Harvey Lectures*, 1905–06, i, 73.
Levene, P. A., and Stookey, L. B., *J. Med. Research*, 1903–04, x, 212.
Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.
Morse, M., *J. Biol. Chem.*, 1917, xxx, 197; xxxi, 303.
Okada, S., *Biochem. J.*, 1916, x, 126.
Opie, E. L., *J. Exp. Med.*, 1905, vii, 316; 1906, viii, 536.
Palitzsch, S., and Walbum, L. E., *Biochem. Z.*, 1912, xlvii, 1.
Parker, J. T., and Franke, E., *J. Med. Research*, 1917, xxxvii, 345.
Ringer, W. E., *Kolloid-Z.*, 1916, xix, 253.
Rona, P., and Arnheim, F., *Biochem. Z.*, 1913, lvii, 84.
Salkowski, *Z. klin. Med.*, 1890, xvii, Suppl., 77.
Schwiening, H., *Virchows Arch. path. Anat.*, 1894, cxxxvi, 444.
Smith, T., *N. Y. Med. J.*, 1894, lx, 590.
Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131.
Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.
Vines, S. H., *Ann. Bot.*, 1904, xvii, 289; 1909, xxiii, 1.
Weinland, E., *Z. Biol.*, 1903, xliv, 45.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1918,
Vol. xxviii, No. 3, pp. 289–296.]

A NOTE ON THE PRODUCTION OF ACID BY PNEUMOCOCCI.

BY GLENN E. CULLEN, PH.D., AND ALAN M. CHESNEY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, May 23, 1918.)

The latent period of growth or "lag," first recognized by Müller in 1895,[1] is the time interval between the inoculation of a medium and the beginning of growth at the maximum rate. Chesney concluded that the latent period in the growth of bacteria is an expression of injury which the bacterial cell has sustained from its previous environment.[2] This conclusion was based upon the observations that the latent period was evident only when the cultures used in inoculation were no longer growing at the maximum rate, and that pneumococci cultures at the end of the period of maximum growth contain a substance that inhibits the growth of actively growing pneumococci.

In studying the nature of the influence which the products of metabolism exert upon the further activity of a microorganism it is important to investigate the changes which may have occurred in the hydrogen ion concentration of the medium. The question of the influence of hydrogen ion concentration has been reviewed exhaustively by Sörensen,[3] by Michaelis,[4] and more recently by Clark and Lubs.[5] Clark and Lubs emphasize its importance as one of the factors controlling bacterial growth thus: "Just as we pay attention to the temperature of the incubator, so should we pay attention to the hydrogen ion concentration of the medium." We therefore undertook to study the hydrogen ion concentration of actively growing pneumococcus cultures.[6]

[1] Müller, M., *Z. Hyg. u. Infectionskrankh.*, 1895, xx, 245.

[2] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

[3] Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.

[4] Michaelis, L., Wasserstoffionenkonzentration. Ihre Bedeutung für die Biologie und die Methoden ihrer Messung, Berlin, 1914.

[5] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

[6] This work is referred to by Chesney.[2]

EXPERIMENTAL.

Bacteriological Technique.—The pneumococcus strains were of Types I and II,[7] which had been isolated several years, passed through many white mice, and transferred innumerable times on artificial media. The beef infusion broth was made by extracting 1 pound of chopped beef with a liter of water over night on ice, filtering, adding peptone to 1 per cent and sodium chloride to 0.5 per cent, and boiling for ½ hour. The reaction was adjusted to 0.2 per cent by the phenolphthalein method, the medium was then boiled 10 minutes, filtered, and sterilized in an Arnold sterilizer for 20 minutes on 3 successive days. One lot of medium was used throughout. The cultures were incubated in the dark in a water bath at 37°C. (+ 1°). The broth was brought to 37°C. before seeding. The number of organisms present in a unit volume was determined by diluting and plating, using 10 to 15 cc. of 1 per cent dextrose agar. The plate method was used in order to count only living organisms.

Chemical Technique.—The hydrogen ion concentrations were determined by the gas chain method with the same precautions used in previous work reported from this laboratory (Cullen[8]). The accuracy of the entire determination was tested frequently by means of Sörensen's standard phosphate and Walpole's[9] standard acetate mixtures of known hydrogen ion concentration.

Since heat or chemical sterilization of the cultures might change the reaction, small samples were removed from the culture flasks, cooled to room temperature, and transferred directly to the electrode vessels where purified hydrogen was admitted and the determination made. Equilibrium is obtained in the Clark electrode in about 5 minutes, so that the time error introduced by this technique is negligible. The apparatus was then chemically sterilized.

Calculation.—It is customary to use the logarithms of the number of organisms in recording results of experiments of rate of growth of bacteria. The logarithms of the viable organism per 0.5 cc. are plotted as ordinates against time intervals as abscissæ. All hydrogen ion

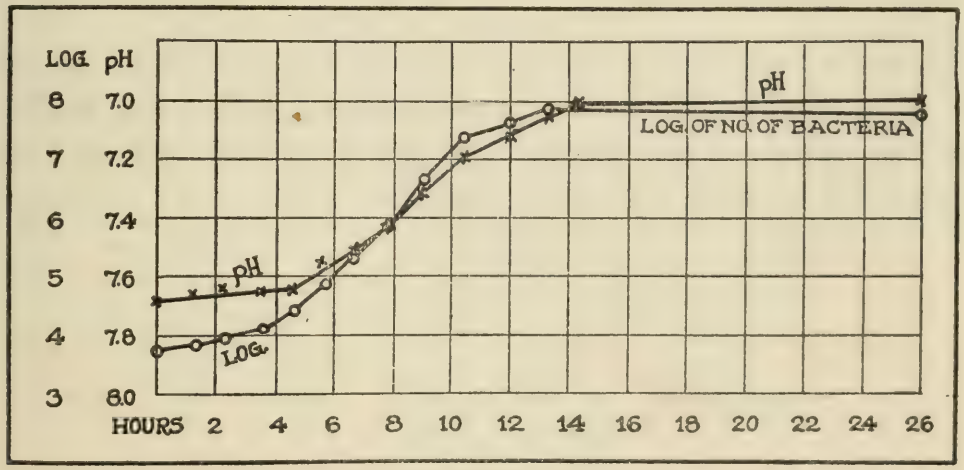
[7] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.
[8] Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 369.
[9] Walpole, G. S., *J. Chem. Soc.*, 1914, cv, 508.

concentration rates are expressed by Sörensen's symbol "pH;" *i.e.*, the Briggs logarithm of the hydrogen ion concentration. In the charts increase in acidity is denoted by a rise in the curve.

Experiment 1.—A flask containing 500 cc. of broth at 37°C. was seeded with a 20 hour culture of Type II. Kept in water bath at 37°C. Samples were removed at the indicated interval for analyses (Table I, Text-fig. 1).

TABLE I.

Time after seeding.	Viable bacteria per 0.5 cc.	Log. of No. of bacteria per 0.5 cc.	Hydrogen ion concentration.
hrs.			*pH*
0	6,750	3.83	7.68
1.3	7,250	3.86	7.66
2.3	9,000	3.95	7.64
3.6	10,800	4.03	7.66
4.6	22,700	4.36	7.65
5.6	79,000	4.90	7.56
6.7	228,000	5.36	7.52
7.8	770,000	5.87	7.44
9.0	4,150,000	6.62	7.33
10.4	23,000,000	7.36	7.20
12.0	38,000,000	7.58	7.12
13.3	71,000,000	7.85	7.05
14.3	74,000,000	7.87	7.02
25.7	61,000,000	7.79	7.00



TEXT-FIG. 1. Pneumococcus Type II. Experiment 1. Curves showing growth and change in hydrogen ion concentration.

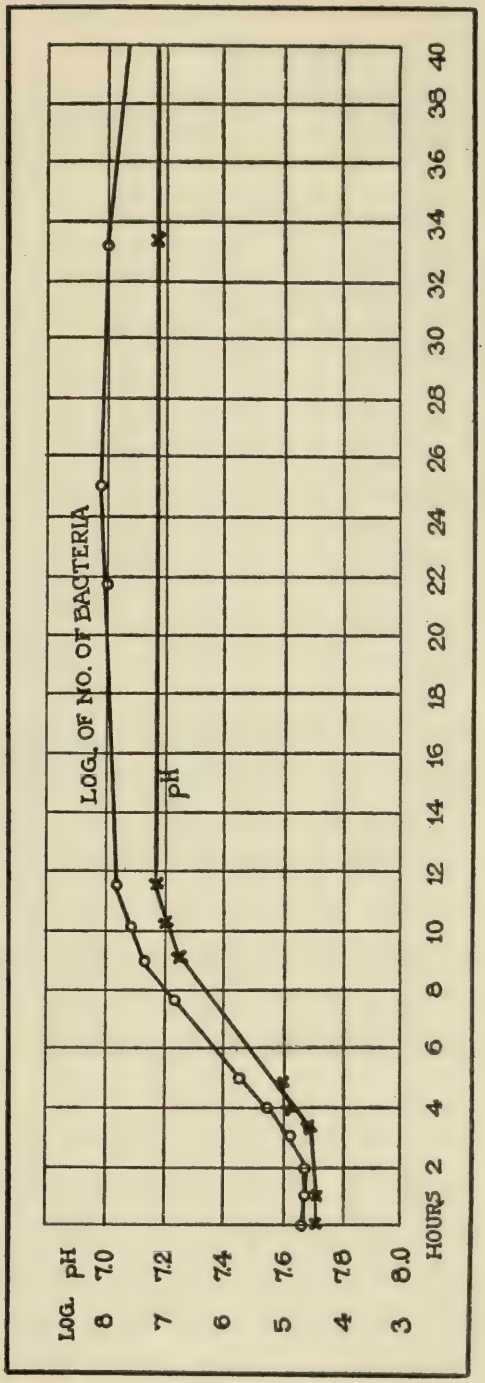
The agreement between these curves is extremely close. The lag in rate of growth parallels the lag in the production of acid products. It should be noted that the change in rate of growth takes place before the change in rate of production of acid.

Experiment 2.—A flask containing 500 cc. of broth at 37°C. was seeded with 0.1 cc. of a 15 hour broth culture of Pneumococcus Type II. Kept in water bath at 37°C. Samples were removed at the indicated interval for analyses (Table II, Text-fig. 2).

TABLE II.

Time after seeding.	Viable bacteria per 0.5 cc.	Log. of No. of bacteria per 0.5 cc.	Hydrogen ion concentration.
hrs.			*pH*
0	43,000	4.63	7.71
1.0	43,400	4.64	7.71
2.0	45,000	4.65	
3.0	84,000	4.92	7.69
4.0	163,000	5.21	7.63
5.0	480,000	5.68	7.60
7.8	7,200,000	6.86	
9.0	20,800,000	7.32	7.24
10.2	33,000,000	7.52	7.21
11.5	72,000,000	7.86	7.17
21.7	108,000,000	8.03	
33.3	126,000,000	8.10	7.17
48.0	20,000,000	7.30	7.15
72.0	1,000	3.00	

These results agree closely with those of Experiment 1. In addition, this experiment was continued further. After the period of maximum growth the hydrogen ion concentration remained fixed although the bacteria were dying rapidly.



TEXT-FIG. 2. Pneumococcus Type II. Experiment 2. Curves showing growth and change in hydrogen ion concentration.

Experiment 3.—A flask containing 500 cc. of broth was seeded with 1 cc. of a 16 hour broth culture of Pneumococcus Type I. Kept in water bath at 37°C. At the indicated interval samples were removed for analyses (Table III, Text-fig. 3).

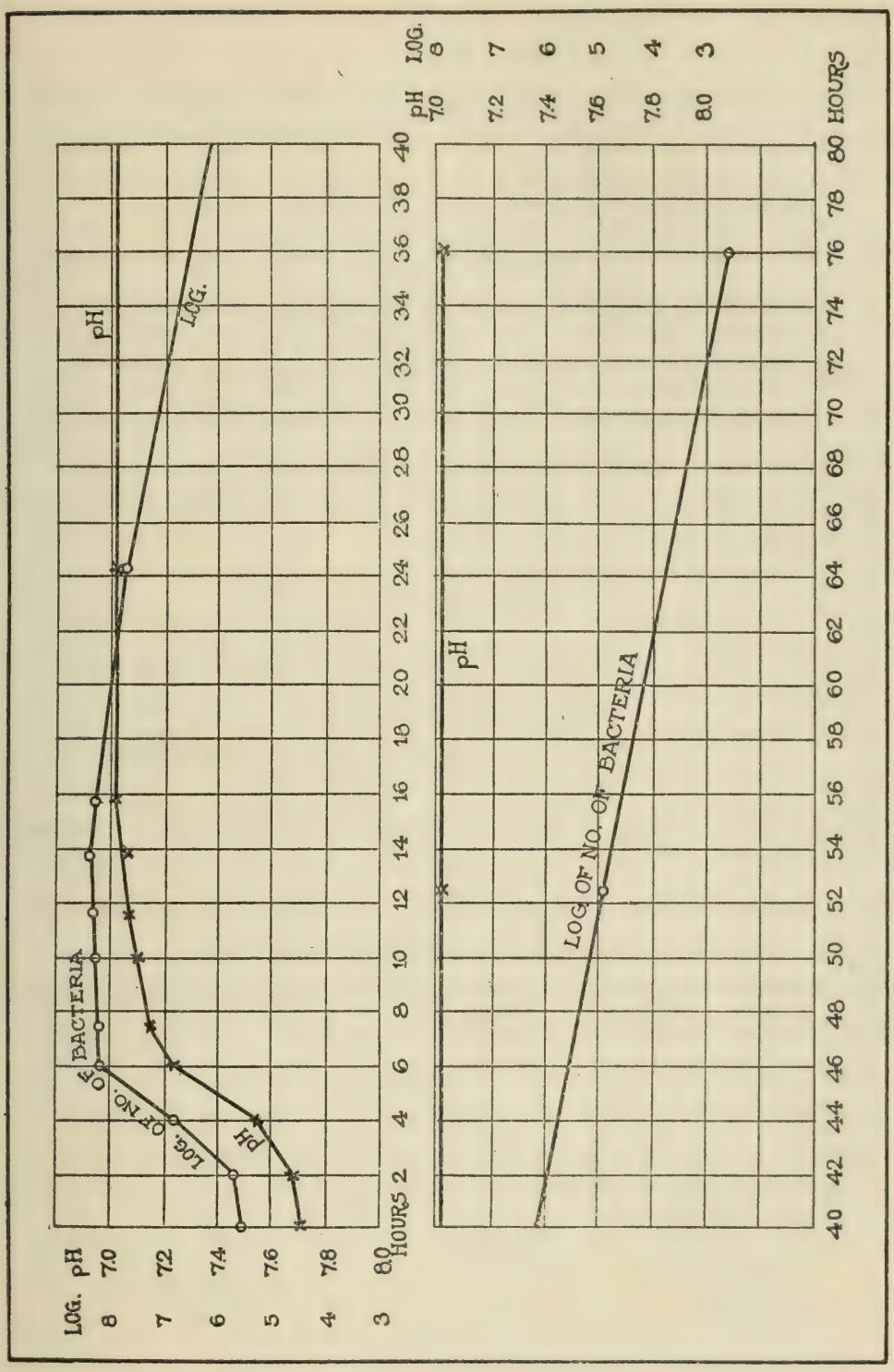
TABLE III.

Time after seeding.	Viable bacteria per 0.5 cc.	Log. of No. of bacteria per 0.5 cc.	Hydrogen ion concentration.
hrs.			*pH*
0	375,000	5.57	7.70
2.0	450,000	5.65	7.68
4.0	6,200,000	6.79	7.56
6.0	130,000,000	8.11	7.24
7.5	145,000,000	8.16	7.14
10.0	160,000,000	8.20	7.11
11.7	177,000,000	8.25	7.09
13.8	210,000,000	8.32	7.07
15.9	166,000,000	8.22	7.03
24.3	57,000,000	7.76	7.03
52.5	63,000	4.80	7.04
76.0	330	2.52	7.04
96.0	80	1.90	7.03

This experiment illustrates the fact emphasized by Chesney that the death rate is comparable with the velocity of a monomolecular reaction, where the concentration of only one substance changes during the reaction. This type of reaction is represented graphically by a straight line curve. It shows, moreover, that the acid production, is a function of the actively growing organism, and that the death of the pneumococcus is not associated with acid production, or with increase in the hydrogen ion concentration.

DISCUSSION.

It is evident from the above results that the pneumococci when growing actively produce acid substance in sufficient quantities to change the hydrogen ion concentration to a marked degree. That this acid production is a metabolic function of the living organism is indicated by the fact that after growth has ceased there is no further change in the hydrogen ion concentration, either while the organisms remain alive, or during the subsequent period when they are dying.



TEXT-FIG. 3. Pneumococcus Type I. Experiment 3. Curves showing growth and change in hydrogen ion concentration.

The fact that cessation of growth occurs always at a pH of about 7 suggests that a hydrogen ion concentration of more than 7 is not favorable to growth. The reaction of the blood, *i.e.* about 7.4, permits the maximum rate of growth. However, the fact reported in the earlier paper (Chesney[2]) that the inhibiting substances are destroyed by incubation for from 4 to 6 days is evidence that the reaction itself is at least not the only cause of the injury, for we have found that the pH does not change during this incubation. Furthermore, since actively growing cultures inoculated into media thus used and incubated, and with a pH of 7, continue to grow, the reaction itself is not sufficiently acid to prevent growth entirely. There must be other inhibiting factors. The nature of these inhibitory substances is at present unknown. Since, however, our present investigation has been indefinitely interrupted, it seems desirable to report it in the present form.

SUMMARY.

1. Actively growing pneumococci produce acid at such a rate that change in the pH of the medium parallels change in rate of growth.

2. The death of the pneumococci is not followed by change in the reaction.

3. Acidification during growth in beef infusion media proceeds until a pH of about 7 is reached. At this point growth stops. The increase in hydrogen ion concentration is not the only origin of the injury to the cell that causes lag in a subsequent culture.

4. The increase in hydrogen ion concentration of the medium is not the sole cause of cessation of growth.

[Reprinted from The Journal of Experimental Medicine, September 1, 1918,
Vol. xxviii, No. 3, pp. 345–357.]

THE OPTIMUM HYDROGEN ION CONCENTRATION FOR THE GROWTH OF PNEUMOCOCCUS.

By K. G. DERNBY, Ph.D., and O. T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 23, 1918.)

It is well known that pneumococcus grows best in slightly alkaline medium, but the exact degree of alkalinity, in terms of hydrogen ion concentration, necessary for optimum growth does not appear to have been determined. The work of Clark (1915) and Clark and Lubs (1917) makes it probable that the limiting and optimum hydrogen ion concentrations for the growth of bacterial races are as definite and characteristic as those for enzyme action. It consequently appears desirable to fix these values for such bacteria as we attempt to cultivate under accurately reproductible conditions. In the work here presented the attempt has been made to ascertain the optimum and limiting hydrogen ion concentrations for the growth of different types of pneumococcus.

Methods.

The substrate was plain salt-free broth, made up as follows: 1 pound of lean chopped beef is allowed to infuse in a liter of tap water over night on ice. The unfiltered meat infusion is boiled for 30 minutes, filtered through paper, and the loss by evaporation made up by the addition of water. 1 per cent peptone is added. The mixture is allowed to boil for 2 minutes. Sufficient sodium hydroxide is then added to turn the solution slightly alkaline to phenolphthalein. The medium is boiled for 6 or 7 minutes, made up to volume, filtered clear, and sterilized in an Arnold sterilizer for 20 minutes on 3 successive days. During this process it becomes less alkaline so that 0.3 to 0.5 cc. of normal sodium hydroxide is required to make 100 cc. neutral to phenolphthalein. The reaction is slightly alkaline, the value for pH lying between 7.6 and 7.9.

Bacterial growth was first estimated by plating. Later it was found that comparative bacterial counts could be made accurately and much more easily by estimating the turbidity of the broth suspensions with a Kober nephelometer. The nephelometric method has already been used by Dreyer and Gardner for this purpose. Its use is limited to solutions with reactions between pH = 6.0 and 8.0, because below a pH of 5.0 some proteins or peptones are precipitated, and above a pH of 8.0 phosphates may precipitate.

We attempted to standardize the method by means of silver chloride suspensions, but they did not prove to be satisfactory standards. The standard solution used was a suspension of dead pneumococci in which the number of cocci per cubic centimeter had been estimated by the plate method before the culture was killed. Because of the difference in color between standard suspension and broth culture, as well as the uncertainty in the absolute count of the standard, the results make no claim to absolute accuracy in the number of bacteria per cubic centimeter. Good comparative results were obtained, however, by comparing all the cultures of a given series with the same standard suspension. The latter was diluted in each instance so that a column of 20 mm. corresponded in turbidity to a column of 15 to 30 mm. of the broth culture.

With a given standard the depth at which the column of a culture must be set in the nephelometer does not vary accurately in inverse ratio to the number of cocci per cubic centimeter of culture. The formula suggested by Kober, however, could not be applied. The relation is expressed by a curve rather than a straight line. This curve was plotted after making a number of control observations in which the standard suspension was compared with known dilutions of itself, and the curve obtained was used as a basis for graphic calculation of results. This is the method that Bloor used in nephelometric determinations of phosphorus.

Hydrogen ion concentrations of the cultures were determined by Sörensen's colorimetric method with some of the modifications suggested by Clark and Lubs. Sörensen's phosphate solutions used as buffers were made by diluting stock 0.5 M solutions of KH_2PO_4 and $Na_2HPO_4.2H_2O$ (Kahlbaum's preparations made according to Sören-

sen). The indicators used were those recommended by Clark and Lubs.[1]

Standard solutions used:

$\frac{M}{15}$ disodium phosphate, $Na_2HPO_4 . 2H_2O$.[2]

$\frac{M}{15}$ monopotassium phosphate, KH_2PO_4.

Indicators used:

Name.	Range of pH.
Dibromo-*o*-cresolsulfonephthalein ("bromocresol purple")	5.2–6.8
Dibromothymolsulfonephthalein ("bromothymol blue")	6.0–7.6
Phenolsulfonephthalein ("phenol red")	6.8–8.4
o-Cresolsulfonephthalein ("cresol red")	7.2–8.8
o-Cresolphthalein	8.2–9.8

For the pH determinations the broth cultures were diluted with two volumes of carbon dioxide-free distilled water, and compared with the standards in the rack shown in Text-fig. 1 which is similar to that used by Walpole and then adopted by Clark and Lubs, but apparently somewhat more convenient. The high color of medium, which in itself might interfere with accurate readings, was compensated by using the comparator method in which the color of a broth control is superimposed upon that of the indicator. The values of pH could be easily determined within a limit of error of 0.05.

All experiments were carried out as follows: Triplicate 5 cc. portions of salt-free broth were placed in test-tubes with 1 cc. portions of buffer solutions to fix the reaction. The tubes were then resterilized. Of each set of three, two were inoculated with 0.1 cc. each of an 18 hour broth culture of pneumococcus while the third served as a control.

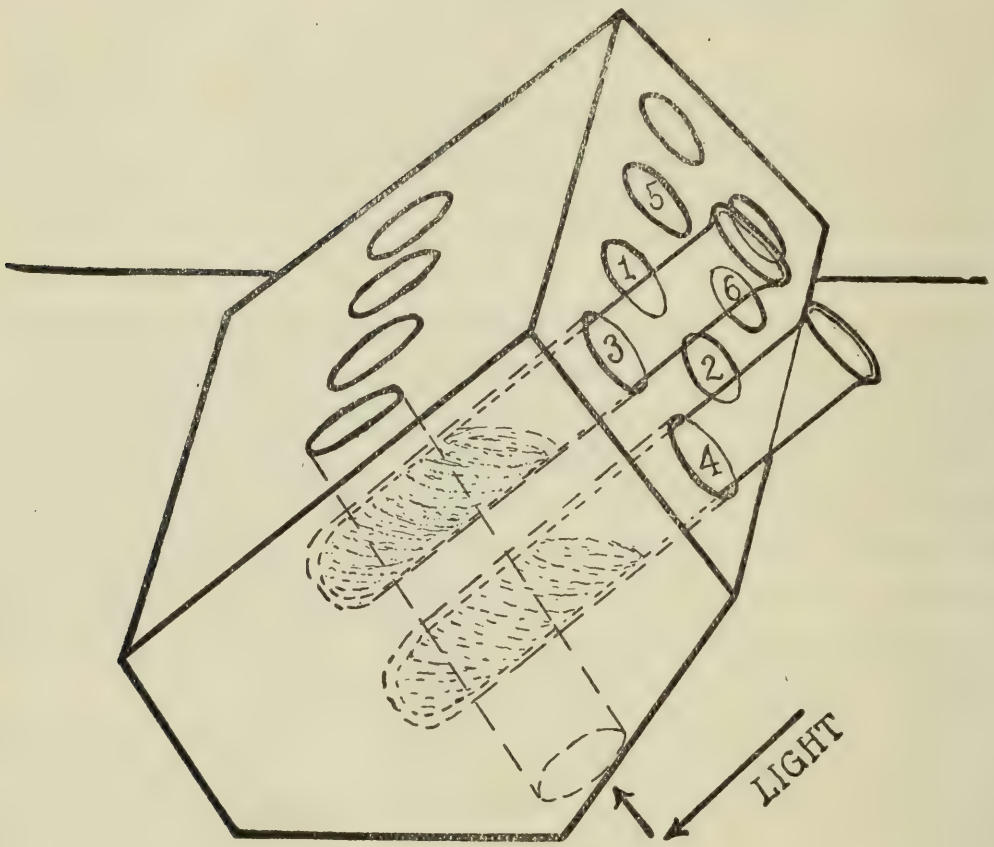
The tubes were incubated at 37°C. for 4 to 48 hours. Portions were taken for bacterial count when the latter was made by plating. The remainder of each culture was then sterilized by heat and the hydrogen ion concentration determined. When the bacterial count was estimated by nephelometer, the entire culture was sterilized before estimating either count or pH.

[1] The indicators were prepared by Hynson, Westcott, and Dunning, Baltimore.

[2] The purity of the salts was verified with the hydrogen electrode by Dr. Glenn E. Cullen.

It may be questioned whether the sterilization by heat in itself affects the hydrogen ion concentration of the broth medium. In order to determine this point the following experiment was devised.

To 5 cc. of uninoculated medium was added 1 cc. of buffer solution as described above. Each solution was then divided into two portions



TEXT-FIG. 1. Walpole's comparator modified to use reflected light. Tube 1, culture; No. 2, water; Nos. 3 and 5, indicator solutions of known hydrogen ion concentration; Nos. 4 and 6, diluted broth controls without indicator.

of 3 cc. each. One portion was sterilized for 20 minutes at 15 pounds pressure in an autoclave and then cooled to room temperature. The other portion served as control. The following results were obtained.

	pH				
After sterilization	7.0	7.4	7.6	8.0	8.8
Control	7.0	7.4	7.6	8.0	8.6

From this experiment it is evident that within the range of pH which we have used in our experiments, no essential change in pH was brought about by sterilization. At greater alkalinities, however, a slight variation seems to occur.

EXPERIMENTAL.

As electrolyte buffers were to be used to fix the hydrogen ion concentrations, it was a necessary preliminary to determine the effect of salt concentration by itself on the growth of pneumococci, in order that buffer concentrations might with certainty be avoided which would retard growth by their mere salt effect or osmotic pressure. Consequently the experiment recorded in Table I was performed.

TABLE I.

Influence of the Salt Concentration on the Growth of Pneumococci (Type I). Sodium and Potassium Chlorides.

Initial pH = 7.9
Incubation: 18 hours at 37°C.

Tube No.	Molar concentration of salt.	Growth.	
		NaCl	KCl
	mols		
1	1.0	−	−
2	0.4	−	−
3	0.2	−+	−+
4	0.1	++	++
5	0.04	++	++
6	0.01	++	++
7	Salt-free broth.	++	++

Concentrations over 0.1 M retard growth, and 0.4 M inhibits it altogether. There seems to be no essential difference in effect between sodium and potassium ions. For this reason we appear justified in using the salts $Na_2HPO_4.2H_2O$ and KH_2PO_4 as reaction regulators without regard to the interchange of potassium and sodium in different solutions.

The results in Table I demonstrate that the osmotic pressure of 0.1 M sodium chloride solution does not retard growth. Since Na_2HPO_4

dissociates into three ions, as compared with the two ions of NaCl, the phosphate solution of corresponding osmotic concentration is approximately $\frac{2}{3} \times 0.1$ M = 0.067 M. In order to stay safely within this limit and rule out osmotic concentration as a factor, the phosphate ion concentration in the cultures was kept down to 0.05 M. The results in Table II indicate that this phosphate concentration is well below that which noticeably retards growth.

TABLE II.

Influence of the Phosphate Concentration on the Growth of Pneumococci (Type I).

Phosphate mixture: 9 parts of Na_4HPO_2 + 1 part of KH_2PO_4.
pH = 7.7.
Incubation: 18 hours at 37°C.

Tube No.	Molar concentration of phosphates.	Growth.
	mols	
1	0.8	−
2	0.4	±
3	0.2	+
4	0.08	++
5	Salt-free broth.	++

TABLE III.

Hydrogen Ion Concentration of Mixtures of Plain Broth, Phosphates, and Hydrochloric Acid or Sodium Hydroxide.

10 cc. of salt-free broth + 1 cc. 0.5 M phosphates + HCl or NaOH + water = 12 cc.

Tube No.	Added.				Hydrogen ion concentration of the mixture.
	0.1 N HCl	0.1 N NaOH	0.5 M KH_2PO_4	0.5 M Na_2HPO_4	
	cc.	*cc.*	*cc.*	*cc.*	*pH*
1	1.2	−	0.5	0.5	6.8
2	0.8	−	0.7	0.3	7.1
3	0.4	−	0.8	0.2	7.3
4	−	−	0.85	0.15	7.5
5	−	−	0.9	0.1	7.7
6	−	0.2	0.93	0.07	7.9
7	−	0.6	0.97	0.03	8.2
8	−	1.2	1.0	0.0	8.6

Table II shows that phosphates by their mere salt effect retard growth in about the same concentration as chlorides.

In Table III the results are recorded of a preliminary experiment performed in order to ascertain the amount of acid, alkali, or phosphate necessary to add to the broth in order to obtain hydrogen ion concentrations over the desired range.

It is known that the pneumococcus in glucose-containing solution is an acid producer. The results in Table IV show the effect of the acid formed on the hydrogen ion concentration of an ordinary broth. The final pH of 6.9 attained after 48 hours is, as shown by later experiments, in itself sufficient to stop growth.

TABLE IV.

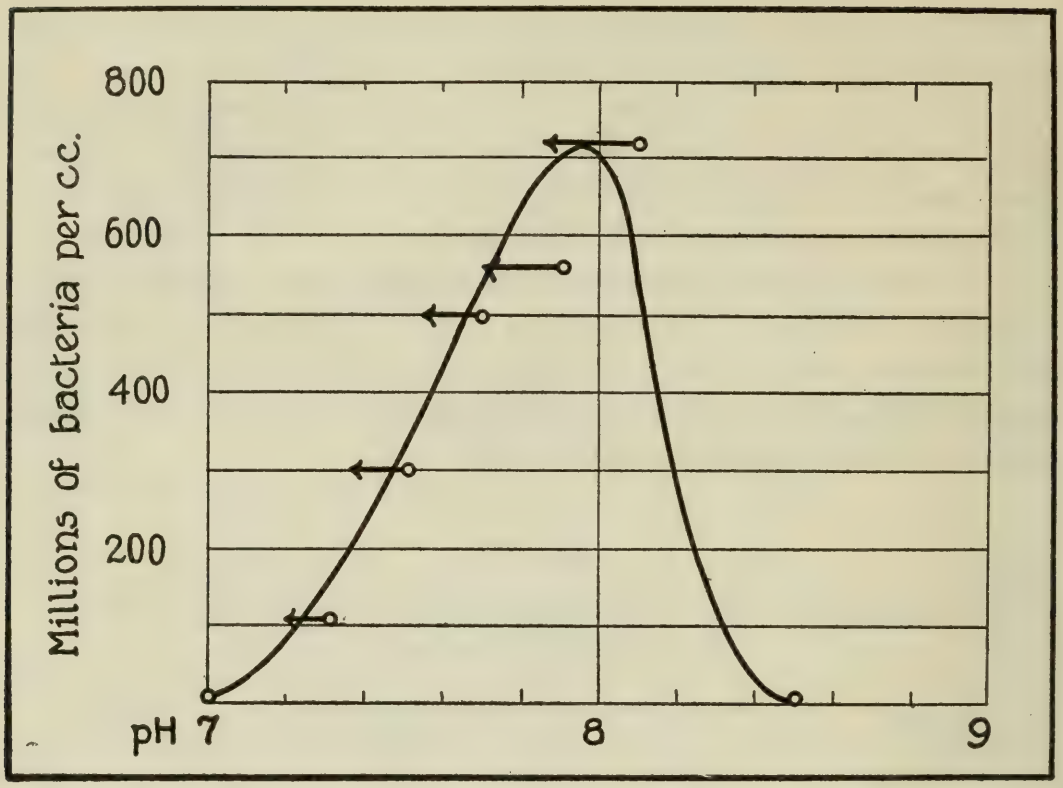
Change in the Hydrogen Ion Concentration of Plain Broth without Buffer Due to the Growth of the Pneumococci.

Tube No.	Length of incubation. (37°C.)	Hydrogen ion concentration.	Growth.
	hrs.	*pH*	
1	3	7.8	−
2	4	7.4	+
3	8	7.35	++
4	24	7.2	++
5	48	6.9	++

The results of Table IV show that buffers must be used to prevent changes in pH so great that results cannot be accurately interpreted, while Table II shows that the concentration of the phosphate buffers must not exceed 0.1 M. With further experience it was found desirable to keep the total phosphate concentration down to 0.05 M.

Results obtained in growing different types of pneumococci in solutions of definite pH, as fixed by phosphate buffers and controlled by colorimetric observations, are given in Tables V to X. The results are summarized graphically in Text-fig. 2.

It is evident from the above results that the optimum hydrogen ion concentration for the growth of pneumococci lies at a pH of about 7.8. It seems to be the same for various types of pneumococcus.



TEXT-FIG. 2. Relation between pneumococcus growth and pH of medium. The arrows indicate the shift in pH towards the acid side which occurred during growth, despite the presence of phosphate buffers. This change is much greater in ordinary media, where phosphates are not added.

TABLE V.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type I.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Type I after 11 hours at 37°C.

Incubation: 4 hours at 37°C.

Growth determined by the plate method.

Tube No.	Hydrogen ion concentration.		Growth.
	Initial.	After 4 hrs.	
	pH	*pH*	
1	7.3	7.3	6,000,000 per cc.
2	7.6	7.35	5,000,000 " "
3	7.85	7.65	38,000,000 " "
4	8.1	7.9	25,000,000 " "
5	8.3	8.0	2,000,000 " "
6	8.6	8.4	1,000,000 " "

TABLE VI.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type I.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Type I after 18 hours at 37°C.

Incubation: 20 hours at 37°C.

Growth determined by the plate method.

Tube No.	Hydrogen ion concentration.		Growth.
	Initial.	After 20 hrs.	
	pH	*pH*	
1	6.8	6.8	1,800 per cc.
2	7.0	7.0	1,700 " "
3	7.25	7.15	900,000,000 " "
4	7.45	7.3	400,000,000 " "
5	7.65	7.4	1,700,000,000 " "
6	7.9	7.65	1,400,000,000 " "
7	8.2	7.75	550,000,000 " "

TABLE VII.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type I.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Type I.

Incubation: 5 hours at 37°C.

Growth nephelometrically determined.

Tube No.	Hydrogen ion concentration.		Growth, determined by the nephelometric method.
	Initial.	After 5 hrs.	
	pH	*pH*	
1	6.9	6.9	0
2	7.15	7.15	27,000,000 per cc.
3	7.3	7.3	52,000,000 " "
4	7.5	7.5	73,000,000 " "
5	7.7	7.6	79,000,000 " "
6	7.9	7.8	120,000,000 " "
7	8.3	8.2	0 " "
Control without phosphates....	7.8	7.4	100,000,000 " "

TABLE VIII.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type II.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + HCl or NaOH + 0.1 cc. of bacterial culture + water = 6 cc.

Type II.

Incubation: 18 hours at 37°C.

Growth nephelometrically determined.

Tube No.	Hydrogen ion concentration.		Growth, determined by the nephelometric method.
	Initial.	After 18 hrs.	
	pH	*pH*	
1	6.8	6.8	0
2	7.0	7.0	0
3	7.3	7.15	450,000,000 per cc.
4	7.5	7.3	500,000,000 " "
5	7.7	7.5	800,000,000 " "
6	7.9	7.6	900,000,000 " "
7	8.3	8.3	Tr.
8	8.6	8.5	0
Control without phosphates....	7.8	7.1	700,000,000 " "

TABLE IX.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Types I, II, and III.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + HCl or NaOH + 0.1 cc. of bacterial culture + water = 6 cc.

Incubation: 22 hours at 37°C.

Growth nephelometrically determined.

Tube No.	Initial hydrogen ion concentration.	Type I.		Type II.		Type III.	
		Hydrogen ion concentration after 22 hrs.	Growth per cc.	Hydrogen ion concentration after 22 hrs.	Growth per cc.	Hydrogen ion concentration after 22 hrs.	Growth per cc.
	pH	*pH*		*pH*		*pH*	
1	7.0	7.0	0	7.0	0	7.0	Tr.
2	7.3	7.2	110,000,000	7.15	280,000,000	7.15	310,000,000
3	7.5	7.35	300,000,000	7.3	440,000,000	7.3	440,000,000
4	7.7	7.45	520,000,000	7.4	500,000,000	7.4	520,000,000
5	7.9	7.6	560,000,000	7.6	850,000,000	7.6	680,000,000
6	8.1	7.85	720,000,000	7.85	800,000,000	7.85	500,000,000
7	8.5	8.5	0	8.5	0	8.5	0

TABLE X.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type II.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Incubation: 18 hours at 37°C.

Growth determined nephelometrically.

Tube No.	Initial hydrogen ion concentration.	Type IIa (Jones).		Type II.		Type IIc.	
		Hydrogen ion concentration after 18 hrs.	Growth per cc.	Hydrogen ion concentration after 18 hrs.	Growth per cc.	Hydrogen ion concentration after 18 hrs.	Growth per cc.
	pH	*pH*		*pH*		*pH*	
1	6.8	6.8	0	6.8	0	6.8	0
2	7.1	7.1	120,000,000	7.1	0	7.1	0
3	7.2	7.2	140,000,000	7.2	140,000,000	7.25	Tr.
4	7.5	7.4	160,000,000	7.4	160,000,000	7.4	150,000,000
5	7.65	7.5	160,000,000	7.5	170,000,000	7.5	160,000,000
6	7.85	7.65	200,000,000	7.7	200,000,000	7.65	220,000,000
7	8.15	8.0	180,000,000	8.0	200,000,000	8.0	170,000,000
8	8.5	8.5	0	8.5	0	8.5	0
Control without phosphates.....	7.8	7.3	200,000,000	7.2	130,000,000	7.2	200,000,000

The optimum reaction is thus slightly more alkaline than the normal hydrogen ion concentration of blood. Growth does not occur in media in which the reaction is more acid than indicated by a pH of 7.0, which is practically the neutral point, or in media of alkalinity greater than indicated by a pH of 8.3. The range of hydrogen ion concentration within which pneumococci grow is consequently a narrow one.

The practical conclusion from these results is that the reaction of culture media for pneumococci should be fixed at a pH of 7.8 to 8.0. Broth with a pH less than 7.6 should not be used. In regulating the

reaction, acid or alkali should be added until direct pH determinations show that the proper reaction has been obtained.[3]

If phosphate solutions are used to assist in regulating the reaction, their concentration in the medium should not exceed 0.1 M, and no other salt should be added.

SUMMARY.

1. The optimum hydrogen ion concentration for the growth of the various types of pneumococcus is a pH of about 7.8.

2. The limiting hydrogen ion concentrations for the growth of pneumococcus are a pH of 7.0 and a pH of 8.3.

3. Phosphates used in adjusting reactions of media retard growth if present in a concentration greater than 0.1 molecular.

4. Culture media for pneumococci should, therefore, have an initial reaction between a pH of 7.8 and 8.0 and a total salt concentration not exceeding 0.1 M.

[3] At present, bouillion for growth of pneumococcus is prepared in this laboratory as described under "Methods," with the exceptions that 0.5 per cent of NaCl is added, and sufficient sodium hydroxide to bring the reaction to a pH of 7.8 to 8.0

The standard solutions required for the range within which media for growth of pneumococcus should fall are the following:

		pH
1.	8.8 cc. of $\frac{M}{15}$ Na_2HPO_4 + 1.2 cc. of $\frac{M}{15}$ KH_2PO_4	7.6
2.	9.2 " " $\frac{M}{15}$ Na_2HPO_4 + 0.8 " " $\frac{M}{15}$ KH_2PO_4	7.8
3.	9.5 " " $\frac{M}{15}$ Na_2HPO_4 + 0.5 " " $\frac{M}{15}$ KH_2PO_4	8.0
4.	9.7 " " $\frac{M}{15}$ Na_2HPO_4 + 0.3 " " $\frac{M}{15}$ KH_2PO_4	8.2

Phenol red or cresol red in 0.2 per cent solutions are satisfactory indicators. If the broth samples used for the pH determination are strongly colored, they may be diluted with two volumes of distilled carbon dioxide-free water, without producing any essential change of the hydrogen ion concentration.

This method of standardizing media has already proved itself valuable. It has practically eliminated the frequent irregularity of growth of pneumococcus media the reaction of which formerly was determined only by titration.

BIBLIOGRAPHY.

Bloor, W. R., A method for the determination of "lecithin" in small amounts of blood, *J. Biol. Chem.*, 1915, xxii, 133; A simple method of converting the Duboscq colorimeter into a nephelometer, *ibid.*, 145.

Clark, W. M., The "reaction" of bacteriologic culture media, *J. Infect. Dis.*, 1915, xvii, 109.

Clark, W. M., and Lubs, H. A., The colorimetric determination of hydrogen ion concentration and its applications in bacteriology, *J. Bacteriol.*, 1917, ii, 1.

Dreyer, G., and Gardner, A. D., A general method of estimating the relative turbidity or opacity of fluid suspensions including bacterial emulsions, *J. Biochem.*, 1916, x, 399.

Kober, P. A., Nephelometry in the study of proteases and nucleases, *J. Biol. Chem.*, 1913, xiii, 485.

Sörensen, S. P. L., Ueber die Messung und die Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen, *Biochem. Z.*, 1909, xxi, 131.

Walpole, G. S., Chart presentation on recent work on indicators, *Biochem. J.*, 1911, v, 207.

[Reprinted from The Journal of the American Medical Association, October 12, 1918, Vol. lxxi, No. 15, pp. 1216–1219.]

FACE MASKS IN INFECTIONS OF THE RESPIRATORY TRACT.

By BREWSTER C. DOUST, M.D.,
Captain, M. C., U. S. Army,
and ARTHUR BATES LYON, M.D.,
Lieutenant, M. C., U. S. Army.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

The use of face masks by those whose duties necessitate their coming in contact with cases of respiratory infection has now become general. The object of such masks is twofold: first, to protect the wearer against infectious material from the respiratory passages of the patient; and second, to protect the patient from such material as the attendant may himself carry in his mouth and nose. The types of masks worn, however, have been variable, and the efficiency of many of these types as barriers to the transmission of bacteria is certainly open to question. We have therefore undertaken experiments with some of the commoner types of masks in order to prove their efficiency or nonefficiency in preventing the dissemination of infectious material from the mouth during the acts of speaking or coughing.

Our first object was to determine, by means of agar plates exposed at different distances from an observer, how far infectious material might be projected from the mouth during speaking or coughing.

Our second object was to determine to what degree the wearing of a mask modified the projection of such material during the same acts.

Methods and Technic.

B. prodigiosus was selected as a suitable organism to introduce into the mouth for these experiments, because of its innocuous character, and because its pigment formation makes its recognition easy on agar plates.

For our experiments two small rooms were selected which were used for no other purpose during this time. Each of these rooms contained a metal topped table and a chair. The arrangement of the furniture is shown in Figure 1. When the observer was seated in the chair, the table top was just below his ensiform. Agar plates exposed on the surface of the tables before the beginning of the experiments showed no *B. prodigiosus* present in the circulating air and dust.

TABLE 1.

*Projection of B. prodigiosus from the Mouth on Agar Plates During Conversational Speech, Loud Speech, and Coughing.**

	Distance of Plates from Operator in Feet									
	1	2	3	4	5	6	7	8	9	10
Speaking in ordinary conversational tone for 5 minutes	**1**	**0**	**0**	**0**	**0**	**0**	—	—	—	—
	0	**0**	**0**	**0**	**0**	**0**	—	—	—	—
	0	**1**	**0**	**0**	**0**	**0**	—	—	—	—
	13	**2**	**0**	**0**	**0**	**0**	—	—	—	—
Speaking in ordinary conversational tone for 30 minutes	**16**	**0**	**0**	**0**	**0**	**0**	—	—	—	—
Speaking in loud tone for 5 minutes	**72**	**0**	**0**	**0**	**0**	**0**	—	—	—	—
	49	**3**	**2**	**0**	**0**	**0**	—	—	—	—
	0	**0**	**0**	**0**	**0**	**0**	—	—	—	—
	115	**5**	**5**	**1**	**0**	**0**	—	—	—	—
Coughing for 5 minutes	**387**	**381**	**245**	**76**	**26**	**4**	—	—	—	—
	—	—	—	**248**	**116**	**94**	**32**	**18**	**17**	**8**
	242	**64**	**19**	**5**	**3**	**1**	—	—	—	—
	—	—	**4**	**6**	**3**	**1**	**2**	**0**	**0**	**0**

* Number of colonies of *B. prodigiosus* on each plate in boldface figures; dash indicates plates not exposed.

Experiment 1. To determine the limits of projection of B. prodigiosus from the mouth during ordinary speech, loud speech, and coughing.—The observer first thoroughly rinsed his mouth and gargled his throat with a suspension of *B. prodigiosus* in 0.85 per cent. sodium chlorid solution, the viability of which was proved by subcultures at suitable intervals. The observer then entered the room and seated himself in the chair, facing down the length of the table. In line with the observer's mouth, agar plates were exposed from 1 to 6 feet distant, except in some of the coughing experiments, when plates were exposed up to a distance of 10 feet.

The observer then proceeded: (1) to talk in an ordinary conversational tone for five minutes; or (2) to talk in a loud tone for five minutes; or (3) to cough as much as possible for five minutes. In one instance ordinary conversational speech was maintained for a period of thirty minutes. At the conclusion of his speaking or coughing, the observer left the room. In order to allow the droplets in the air, if present, to settle on the surface of the plates, the plates remained exposed an additional ten minutes after the period of speaking or coughing. The colonies occurring on the plates were counted and recorded after seventy-two hours' incubation at room temperature.

Agar plates were exposed for one hour in the rooms between different stages of this experiment. By this means we found that an eight hour interval between the periods of talking or coughing was sufficient to insure the absence of *B. prodigiosus* in the circulating air.

The results of the projection of *B. prodigiosus* from the uncovered mouth during ordinary conversational speech, loud speech, and coughing, are shown in Table 1. The experiments were paralleled by two observers working in separate rooms.

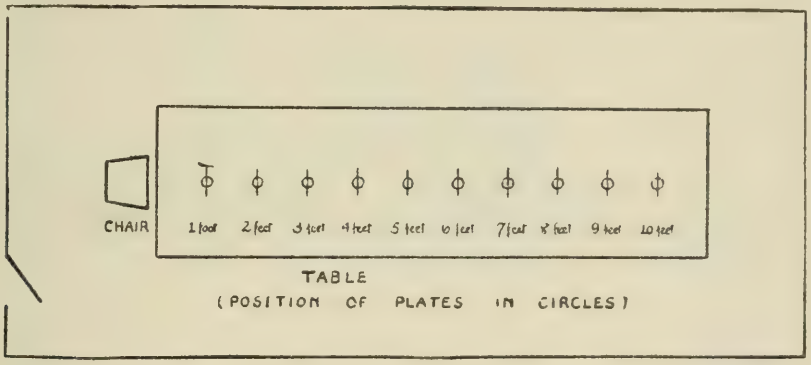


FIG. 1.—Arrangement of furniture and plates for tests.

Summary of Table 1.

1. Ordinary Conversational Speech for Five Minutes.—In three out of four trials, *B. prodigiosus* was recovered in small numbers on plates 1 or 2 feet distant, but no farther.

2. Ordinary Conversational Speech for Thirty Minutes.—One trial. *B. prodigiosus* was recovered only on the 1 foot plate in slightly larger numbers than in any of the preceding experiments.

3. Loud Speech for Five Minutes.—In three out of four trials, *B. prodigiosus* was recovered in moderate numbers on plates from 1 to 4 feet distant.

4. *Coughing for Five Minutes.*—In all of four trials, *B. prodigiosus* was recovered, usually in large numbers, on plates up to 6 feet distant.

In two trials in which plates were exposed up to 10 feet, *B. prodigiosus* was once recovered up to 7 feet, and once up to 10 feet.

Masks.

For the subsequent experiments, masks were used. The masks employed by us were of uniform construction, but differed in the nature of the material used and in thickness.

Three sorts of material were employed: (1) coarse gauze; (2) medium gauze; and (3) buttercloth (Fig. 2). From each sort masks were made of from two to ten layers in thickness.

Each mask was about 6 by 8 inches, hemmed on the edges, and made with four plaits on each lateral edge. The mask was equipped with tapes on the four corners by which to tie it behind the head

TABLE 2.

Projection of B. Prodigiosus from the Mouth while the Face is Covered with a Mask of Coarse Gauze.

Speaking in Ordinary Conversational Tone for 5 Minutes						Number of Layers of Gauze in Mask	Speaking in Loud Tone for 5 Minutes						Number of Layers of Gauze in Mask	Coughing for 5 Minutes					
Distance of Plates from Operator in Feet							Distance of Plates from Operator in Feet							Distance of Plates from Operator in Feet					
1	2	3	4	5	6		1	2	3	4	5	6		1	2	3	4	5	6
0	0	0	0	0	0	2	75	1	0	0	0	0	2	700	800	610	397	128	28
0	0	1	0	0	0	3	18	1	0	2	0	0	3	125	84	31	5	4	2
0	0	0	0	0	0	4	22	1	0	0	0	0	4	400	280	126	35	8	3
0	0	2	0	0	0	5	2	0	0	0	0	0	5	140	160	100	42	15	1
0	0	0	0	0	1	6	5	0	0	0	0	0	6	65	20	10	4	0	1
0	0	0	0	0	0	7	3	0	0	0	0	0	7	68	63	15	0	0	0
0	1	0	0	0	0	8	2	0	0	0	0	0	8	8	1	0	0	0	0
0	0	0	0	0	0	9	0	0	0	0	0	0	9	6	4	2	0	0	0
0	0	0	0	0	2	10	0	0	0	0	0	0	10	9	2	0	0	0	0

(Fig. 3). When extended to the full width by a fanlike extension of the plaits, the mask presented sufficient surface to cover nose and mouth, and to come below the chin (Fig. 4).

Experiment 2. To determine the effect of a coarse gauze mask on the projection of B. prodigiosus from the mouth during ordinary speech, loud speech, and coughing.—The methods and technic of this experiment were the same as in Experiment 1, except that during talking or coughing a coarse gauze mask was worn. Each of the experiments of speaking in an ordinary tone, speaking in a loud tone, and coughing, was done with each mask of from two to ten thicknesses of gauze. The results of these experiments are shown in Table 2.

Summary of Table 2.

Coarse gauze masks worn, from two to ten layers thick.

1. Ordinary Conversational Speech for Five Minutes.—One trial with each mask. *B. prodigiosus* was recovered five times, always in small numbers, on plates at variable distances. Three times organisms were found at 2 to 3 feet and twice at 6 feet. The latter possibly represents an error, as even without masks we never recovered organisms at this distance during ordinary speech. The error, if present, is insignificant, since the remaining data are conclusive.

2. Loud Speech for Five Minutes.—With each mask, one trial. *B. prodigiosus* was recovered on plates from 1 to 4 feet distant with every mask up to eight thicknesses—in fairly large numbers with those up to four thicknesses, and in small numbers with those of from five to eight thicknesses.

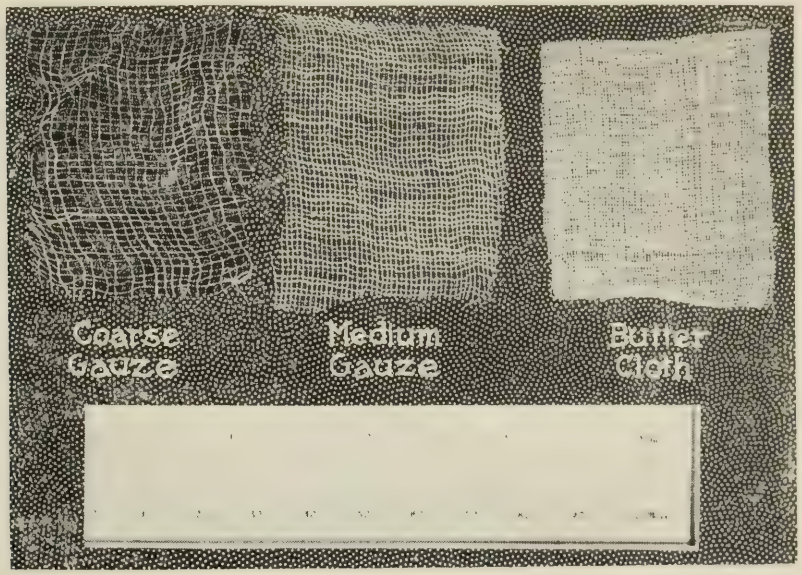


FIG. 2.—Material employed for masks.

3. Coughing for Five Minutes.—With each mask, one trial. *B. prodigiosus* was recovered in every instance. It was recovered in large numbers with masks of six thicknesses or less, up to 6 feet. With masks of greater thickness it was recovered in larger or smaller numbers at a distance of 2 or 3 feet.

Experiment 3. To determine the effect of a medium gauze mask in the projection of B. prodigiosus from the mouth during ordinary speech, loud speech, and coughing.—The methods and technic of this experiment were exactly the same as in the preceding, except that the masks used were of a closer meshed gauze. The experiments of talking in an ordinary tone, talking in a loud tone, and coughing were repeated with each mask of from two to five layers in thickness.

Since the masks of from three to five layers proved to be effective as a barrier to organisms during ordinary speech, this experiment was abandoned with the

thicker masks. Only loud talking and coughing were attempted with the masks of from six to ten layers.

The results of this experiment are shown in Table 3.

Summary of Table 3.

Medium gauze masks worn, from two to ten layers thick.

1. Ordinary Conversational Speech for Five Minutes.—With each mask of from two to five layers, one trial. *B. prodigiosus* was recovered in small numbers only on the 1 foot plate with the two layer mask.

TABLE 3.

Projection of B. prodigiosus from the Mouth while the Face is Covered with a Mask of Medium Gauze.

Speaking in Ordinary Conversational Tone for 5 Minutes						Number of Layers of Gauze in Mask	Speaking in Loud Tone for 5 Minutes						Number of Layers of Gauze in Mask	Coughing for 5 Minutes					
Distance of Plates from Operator in Feet							Distance of Plates from Operator in Feet							Distance of Plates from Operator in Feet					
1	2	3	4	5	6		1	2	3	4	5	6		1	2	3	4	5	6
5	0	0	0	0	0	2	220	4	0	0	0	0	2	15	6	1	1	1	0
0	0	0	0	0	0	3	1	0	1	0	0	0	3	39	25	12	2	1	0
0	0	0	0	0	0	4	0	0	0	1	0	0	4	45	21	8	1	0	0
0	0	0	0	0	0	5	0	0	0	0	0	0	5	2	1	0	0	0	0
—	—	—	—	—	—	6	1	0	0	0	0	0	6	0	1	0	0	0	0
—	—	—	—	—	—	7	2	0	0	0	0	0	7	8	10	7	9	7	6
—	—	—	—	—	—	8	0	0	0	0	0	0	8	5	0	1	1	1	0
—	—	—	—	—	—	9	0	0	0	0	0	0	9	3	6	1	1	4	1
—	—	—	—	—	—	10	0	0	0	0	0	0	10	1	0	0	1	0	0

2. Loud Speech for Five Minutes.—With each mask of from two to ten layers, one trial. *B. prodigiosus* was recovered in five cases, never beyond 4 feet, usually in small numbers. The 1 foot plate with the two layer mask was heavily seeded.

3. Coughing for Five Minutes.—With each mask of from two to ten layers, one trial. In every instance, *B. prodigiosus* was recovered in moderate numbers on plates from 2 to 6 feet distant.

Experiment 4. To determine the effect of a buttercloth mask on the projection of B. prodigiosus from the mouth during ordinary speech, loud speech, and coughing.—The methods and technic of this experiment were the same as used in the preceding, but the masks used were made of buttercloth. Ordinary speech, loud speech, and coughing were repeated with each mask of from two to ten layers in thickness.

The results of these experiments are shown in Table 4.

Summary of Table 4.

Buttercloth masks worn, from two to ten layers thick.

1. Ordinary Conversational Speech for Five Minutes.—With each mask, one trial. *B. prodigiosus* not recovered.

2. Loud Speech for Five Minutes.—With each mask, one trial. *B. prodigiosus* not recovered.

3. Coughing for Five Minutes.—With each mask, one trial. *B. prodigiosus* recovered at 2 feet with the two layer mask only.

Repetition of the coughing experiments while two or three layer masks, both washed and unwashed, were worn, showed like results.

TABLE 4.

Projection of B. Prodigiosus from the Mouth while the Face is Covered with a Mask of Buttercloth.

Speaking in Ordinary Conversational Tone for 5 Minutes						Number of Layers of Gauze in Mask	Speaking in Loud Tone for 5 Minutes						Number of Layers of Gauze in Mask	Coughing for 5 Minutes					
Distance of Plates from Operator in Feet							Distance of Plates from Operator in Feet							Distance of Plates from Operator in Feet					
1	2	3	4	5	6		1	2	3	4	5	6		1	2	3	4	5	6
0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	2	0	0	0	0
0	0	0	0	0	0	3	0	0	0	0	0	0	3	0	0	0	0	0	0
0	0	0	0	0	0	4	0	0	0	0	0	0	4	0	0	0	0	0	0
0	0	0	0	0	0	5	0	0	0	0	0	0	5	0	0	0	0	0	0
0	0	0	0	0	0	6	0	0	0	0	0	0	6	0	0	0	0	0	0
0	0	0	0	0	0	7	0	0	0	0	0	0	7	0	0	0	0	0	0
0	0	0	0	0	0	8	0	0	0	0	0	0	8	0	0	0	0	0	0
0	0	0	0	0	0	9	0	0	0	0	0	0	9	0	0	0	0	0	0
0	0	0	0	0	0	10	0	0	0	0	0	0	10	0	0	0	0	0	0

Comment.

In 1899, Flügge and his followers,[1] working on the problem of tuberculosis infection, first advanced the theory of droplet infection. Briefly speaking, their experiments showed that if an observer retained in his mouth a suspension of *B. prodigiosus*, the organism could be recovered on agar plates a meter distant during the acts of speaking, sneezing, or coughing. In general they believed that if a

1. Flügge: Ztschr. f. Hyg., 1899, 30, 107. Laschschenko: Ibid., p. 125. Heyman: Ibid., p. 139. Hillyer: Brit. Med. Jour., 1903, 1, 595.

person were coughed at from a distance of less than a meter, he might be infected by means of the projected droplets.

From our own work, however, we judge that the infected zone has a considerably greater radius. Our data indicate that during ordinary or loud speech, droplets are rarely projected more than 4 feet, irrespective of the duration of the experiment. In talking for thirty minutes there was no greater projection of bacteria than in talking for five minutes, though during the longer time there may be a heavier seeding of a plate within the effective range. During coughing, however, the danger zone is immensely widened. We were surprised at the distance we could project the organisms during a hard

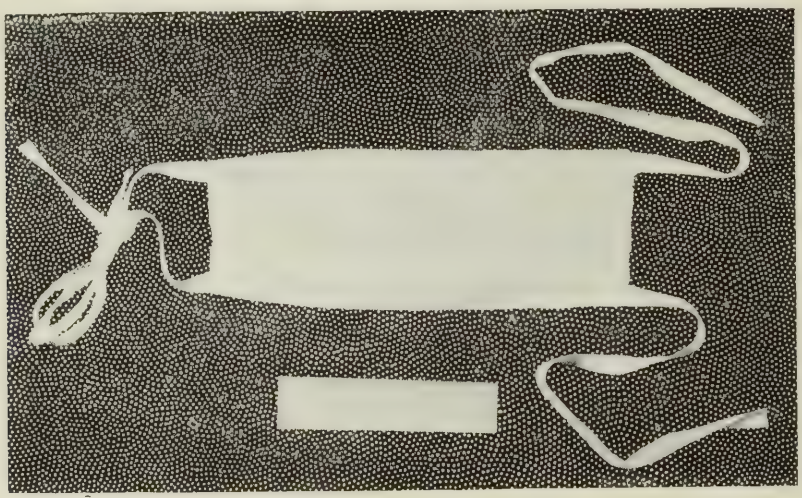


FIG. 3.—Mask, with tapes.

spell of coughing. At first we exposed plates only to a distance of 6 feet. Subsequently we exposed plates up to a distance of 10 feet, on which we recovered *B. prodigiosus* in sufficient numbers to suggest the possibility of even greater projection. We found that the chances of recovering *B. prodigiosus* from the more distant plates were increased if the observer renewed the organisms in his mouth during the period of coughing. From our work it would seem that the danger zone about a coughing patient has at least a 10 foot radius.

Our work has also convinced us of the futility of masks constructed from the coarser types of gauze, irrespective of thickness. We were constantly able to cough organisms through ten thicknesses

of such material. A three layer buttercloth mask, however, appears to be effective in preventing the distribution of organisms from the mouth even during violent coughing. We have repeated experiments with this mask both before and after washing, with uniform results. Such a mask is not only effective, but easy to make and perfectly comfortable to wear.

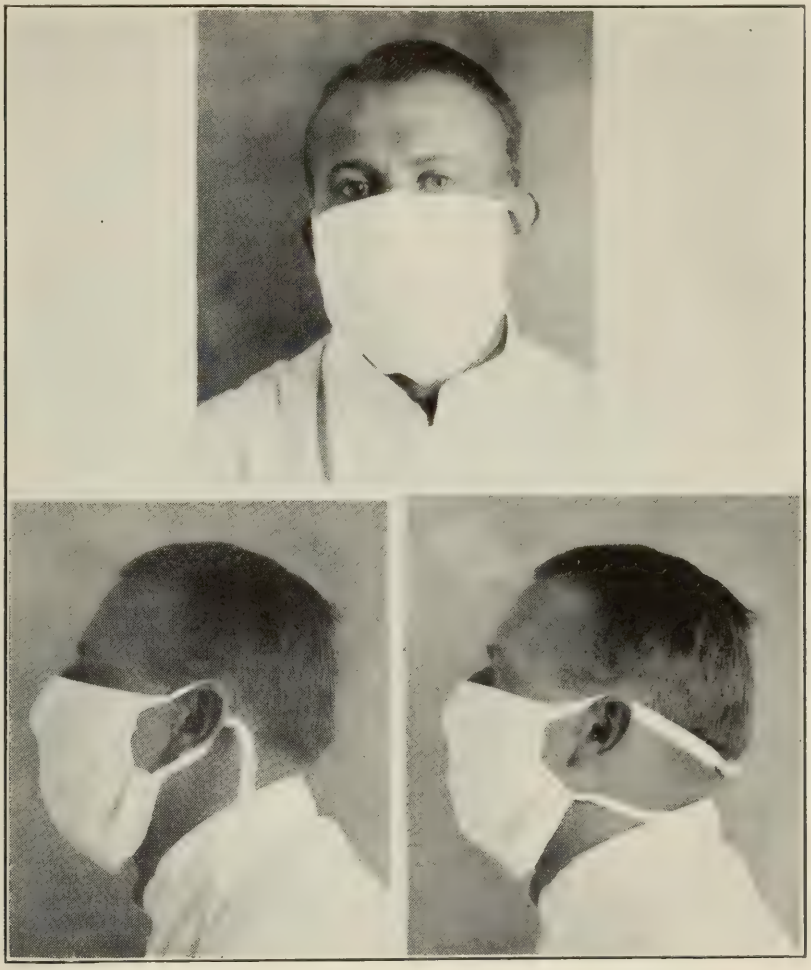


FIG. 4.—The mask as worn.

These masks are also inexpensive. The quoted price per square yard of buttercloth is 14 cents. A square yard makes nine masks, each three layers in thickness. Each mask requires fifty-six inches of tape, costing about 2 cents. The cost per mask therefore is only between 3 and 4 cents, exclusive of labor. A mask may be repeatedly washed and used indefinitely. An indelible mark should be made on

one side of the mask, and when the mask is put on, the same side should always be next to the face.

We have been using these masks in the Hospital of The Rockefeller Institute for physicians and nurses, and in some instances for patients; for example, during carriage to and from the roentgen-ray room, or when being moved about the corridors. They have also been used for ambulatory patients with head colds or other mild respiratory infections.

CONCLUSIONS.

1. During ordinary or loud speech, infected material from the mouth is rarely projected to a distance of 4 feet, and usually less. A 4 foot danger zone exists about the patient under these conditions.

2. During coughing, infected material from the mouth may be projected at least 10 feet. The danger zone about a coughing patient has, then, a minimum radius of 10 feet.

3. Masks of coarse or medium gauze of from two to ten layers do not prevent the projection of infected material from the mouth during coughing. Such masks are worthless, therefore, in preventing the dissemination of respiratory infections.

4. A three layer buttercloth mask is efficient in preventing the projection of infectious material from the mouth during speaking or coughing. It is a suitable mask, therefore, to be worn in connection with respiratory diseases.

[Reprinted from The Journal of Biological Chemistry, November, 1918, Vol. xxxvi, No. 2, pp. 351–354.]

STUDIES OF ACIDOSIS.

XI. The Determination of Carbon Dioxide in Carbonates.

By DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 16, 1918.)

The method described below was devised primarily for use in determination of the carbonate in bones which had been dried and pulverized, but not ashed. This material offered peculiar difficulties to analysis by the usual methods. The method adopted appears to be applicable to all carbonates, soluble or insoluble, in the absence of acids, such as hydrogen sulfide, that are highly volatile from water solution. The principle of rapid extraction of carbon dioxide from solution by means of reduced pressure, utilized in the author's method for determining bicarbonates in blood[1] has been combined with the familiar precipitation of carbonic acid by standard barium hydroxide solution and titration of the excess of hydroxide. Under the reduced pressure the transfer of carbon dioxide to the barium hydroxide solution can be completed in 3 minutes, and the fact that the entire process is carried out in a single closed vessel practically excludes error from loss of carbon dioxide.

The carbonate, either pulverized or in solution is placed in the bottom of a tube 20 to 25 mm. in diameter which is placed in a 250 cc. suction flask (Fig. 1) containing an excess of 0.1 N barium hydroxide. When pulverized carbonate is weighed into the tube, care must be taken that the entire amount is placed at the bottom, as portions sticking to the walls may fail to be decomposed by the acid added later. Our practice has been to weigh the tube, slip into it a roll of glazed paper to protect the walls, pour in the substance, and reweigh the tube after withdrawing the paper. When

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

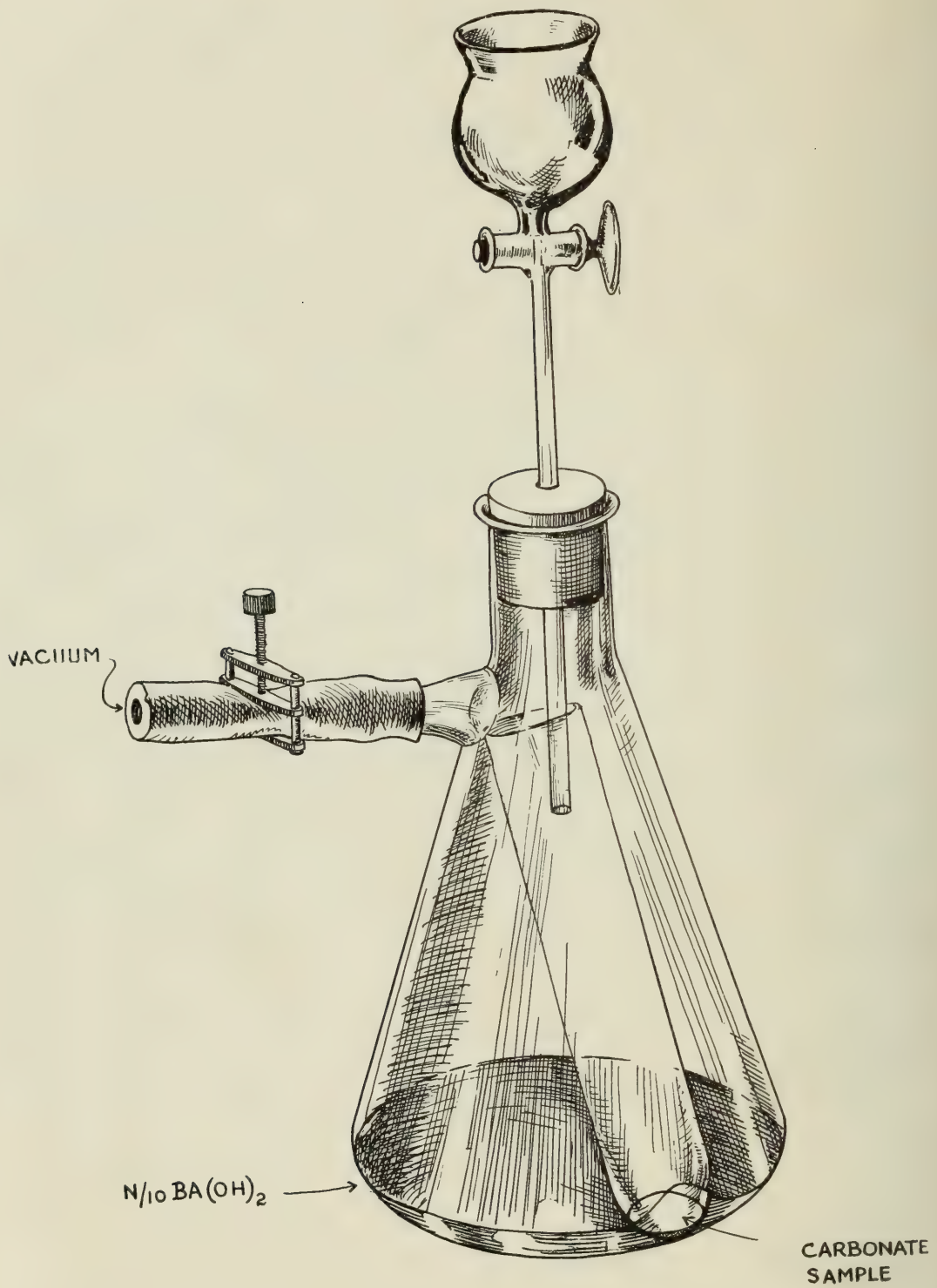


FIG. 1.

the tube is in place (Fig. 1), the flask is evacuated to a pressure of 50 mm. or less, and the outlet is closed with the screw clamp. An excess of normal hydrochloric acid, usually about 5 cc., is admitted slowly from the dropping funnel. Some of the solution is left above the stopper to assure its remaining air-tight. When the rapid evolution of carbon dioxide has ceased, the solutions are both agitated by a rotary motion for 3 minutes. In the analysis of sodium or calcium carbonate, this period has proved sufficient for complete transfer of carbon dioxide from the inner tube to the barium hydroxide solution. If the solution is not agitated, however, so that the surface of the barium hydroxide solution is continually renewed, the flask may stand for an hour without complete absorption of the carbon dioxide.

In the analysis of bones, which have been pulverized but not ashed, time must be allowed for the acid to penetrate into the particles, which are permeated with fat and protein. In this case it has been found necessary to allow at least 2 hours instead of 3 minutes for complete evolution and absorption of carbon dioxide. In routine analyses we have made a practice of allowing 5 hours. The solutions in the flask are stirred by rotation occasionally during this period, and for 3 minutes at the end of it.

After the reaction is completed the vacuum is released, and the barium carbonate is removed by filtration with a Gooch crucible, flask and crucible being rinsed with 3 portions of about 20 cc. each of water. The filtrate in the receiving suction flask is titrated against 0.1 N hydrochloric acid using phenolphthalein as indicator.

EXPERIMENTAL.

Sodium Carbonate.—A sodium carbonate solution was prepared containing 10 gm. of Merck's anhydrous Na_2CO_3 per 100 cc. Titration with methyl orange indicated that the solution was of the calculated concentration. 1 cc. portions, measured to within 0.001 cc. with an Ostwald pipette, required 18.93 and 18.87 cc. of 0.1 N HCl, the calculated amount being 18.85.

Calcium Carbonate.—The calcium carbonate used was a Baker and Adamson's C. P. precipitated. To check its purity varying

amounts were dissolved in excess 0.1 N hydrochloric acid, the carbon dioxide was expelled by boiling, and the excess acid titrated back with 0.1 N barium hydroxide, using alizarin sulfonate as indicator. The 0.1 N acid had been prepared by the method of Hulett and Bonner.[2]

TABLE I.

$CaCO_3$	0.1 N HCl	0.1 N $Ba(OH)_2$	0.1 N HCl neutralized by $CaCO_3$.		$CaCO_3$ in preparation used.
			Observed.	Calculated.	
gm.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*
0.4424	100.00	12.20	87.80	88.48	99.3
0.2060	50.00	9.29	40.71	41.20	98.8
0.2105	50.00	8.36	41.64	42.10	98.9
Average.....					99.1

In accordance with the above results, the weights of calcium carbonate taken in the following analyses were corrected by multiplication by the factor 0.991, and the weights given in Table II are so corrected.

TABLE II.

Determination of Carbon Dioxide in Sodium Carbonate Solution and in Pulverized Calcium Carbonate.

Na_2CO_3 (in 10 per cent solution).	$CaCO_3$	0.1 N $Ba(OH)_2$ in flask.	0.1 N HCl to titrate excess $Ba(OH)_2$.	0.1 N $Ba(OH)_2$ precipitated by CO_2.	CO_2 found.	CO_2 present.
gm.	*gm.*	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*
0.100	—	29.00	10.20	18.80	0.0414	0.0415
0.100	—	29.00	10.15	18.85	0.0415	0.0415
0.100	—	29.00	10.20	18.80	0.0414	0.0415
—	0.0730	20.28	5.55	14.73	0.0324	0.0321
—	0.0909	20.28	2.00	18.25	0.0402	0.0400
—	0.1652	40.56	7.50	33.06	0.0727	0.0726
—	0.1709	40.56	6.20	34.36	0.0756	0.0752
—	0.1764	40.56	5.45	35.11	0.0773	0.0778

The accompanying paper by Dr. Goto gives the results obtained in bone analysis.

[2] Hulett, G. A., and Bonner, W. D., *J. Am. Chem. Soc.*, 1909, xxxi, 390.

[Reprinted from The Journal of Biological Chemistry, November, 1918, Vol. xxxvi, No. 2, pp. 355–376.]

STUDIES OF ACIDOSIS.

XII. Mineral Metabolism in Experimental Acidosis.

By KINGO GOTO.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 16, 1918.)

No detailed study of the mineral changes in the soft tissues and skeleton respectively, caused by either clinical acidosis or prolonged acid feeding, has been published so far as we have been able to ascertain. We have only the observation which Gerhardt and Schlesinger (1899) report to have been made by von Frerich, that the skeletons of diabetics dying in coma appear to be atrophied. Quantitative work on the subject appears to have been limited chiefly to a study of excretion.

Knowledge derived from direct analysis of the body itself has been limited to the blood. Walter (1877) showed that entrance of acids into the circulation causes an immediate reduction of the blood bicarbonate, and Palmer and Van Slyke (1917) have found that the bicarbonate concentrations of the body fluids in general parallel those of the blood plasma, so that depletion of bicarbonate in the blood plasma indicates a depletion of the bicarbonate stores throughout the body. When such a bicarbonate deficit continues, some depletion of the other alkali reserves might also be expected, and the literature contains a considerable amount of metabolic work which indicates that it occurs.

Ash analyses indicate that the main reserves of alkali aside from the bicarbonates are the calcium carbonate and the much greater amount of calcium phosphate in the bones, and the potassium and sodium phosphates of the tissues; *i.e.*, the alkali reserves aside from the carbonates are chiefly phosphates. It would therefore be expected that if the reserves of either the skeleton or soft tissues are drawn upon to a considerable extent, an increased excretion of phosphates would result, chiefly sodium and potassium phosphates if the

source were the soft tissues, calcium phosphate if it were the bones. Increased phosphate excretion in the urine has in fact been reported in diabetic ketonuria and in experimental hydrochloric acid feeding in man and in animals.

In diabetic ketonuria, however, it is uncertain whether the increased phosphoric acid excretion is due to loss of alkaline phosphates from the body, or merely to combustion of increased amounts of food and body proteins. Gerhardt and Schlesinger (1899) as evidence that there is an excretion of P_2O_5 in excess of that accountable for by protein catabolism, report a $\frac{P_2O_5}{N}$ ratio of $\frac{18-20}{100}$ in the urines of two diabetics with heavy ketonuria. The normal ratio under similar conditions was estimated at $\frac{12.5}{100}$ Feeding sodium bicarbonate to the diabetics reduced it to $\frac{14-16}{100}$. Rumpf (1898) and Mandel and Lusk (1904) report in a fasting patient with severe diabetes $\frac{P_2O_5}{N}$ ratios similar to those of Gerhardt and Schlesinger. Folin and Shaffer (1902), however, found similar ratios, $\frac{18-20}{100}$, common in normal adults. Consequently, as stated above, the data at hand do not tell us whether or not the alkaline phosphates of the body are depleted in diabetic acidosis.

Increased excretion of calcium and magnesium in the urine has been reported in diabetic acidosis by Gerhardt and Schlesinger (1899) and by Tenbaum (1896). Lack of complete intake and outgo data, however, makes it impossible to state whether the figures indicate a real loss of bone substance.

The metabolic experiments with acid feeding in the literature include observations more comprehensive than were made in the above cited studies on diabetics. Steenbock, Nelson, and Hart (1914) found that feeding hydrochloric acid to a calf increased the urinary phosphoric acid, but that even large doses showed no tendency to produce a negative phosphate balance. When the maximum dose of 400 cc. of 1 N HCl per day was given, a negative calcium balance was produced. The authors conclude that

the acid attacked only the calcium carbonate, not the calcium phosphate of the bones.

Fitz, Alsberg, and Henderson (1907) observed in the urine of rabbits, which received dilute hydrochloric acid daily, a marked increase of phosphoric acid excretion, followed by a decrease, presumably when the readily available phosphates were exhausted. In some cases the excretion rose again before death.

Givens and Mendel (1917) examined the balance of nitrogen, calcium, magnesium, and phosphorus after the administration of base and acid in the dog. The phosphorus balance showed no significant effect and was always positive, though extremely variable quantitatively. The figures for both the urinary and the fecal phosphorus were so variable that neither could be used as an index of any influence exerted by sodium bicarbonate administration. Stehle (1917) states that the administration of hydrochloric acid by mouth to the dog causes an increased excretion of calcium and magnesium as well as sodium and potassium.

Except for the results of Givens and Mendel (1917, 1918) with dogs, the above literature indicates that sufficient acid feeding produces in various animals an increased excretion of phosphate in the urine. The results of Fitz, Alsberg, and Henderson (1907), who followed the daily P_2O_5 excretion of rabbits fed with HCl for weeks until death resulted, are particularly significant.

In our experiments the chief object has been to ascertain the effect of acid feeding on the mineral composition of the bones and muscles respectively; but we have followed the phosphate excretion in the urines of our rabbits in order to make certain that the animals were actually responding with increased phosphate excretion in the manner to be expected from the experiments of Fitz, Alsberg, and Henderson. The results were uniformly confirmatory.

There are, however, two possible explanations of the increased phosphate excretion in the urine caused by acid feeding. The increased urinary phosphate may be drawn from the reserves of the body to neutralize the acid, or it may be due merely to increased absorption of calcium and magnesium phosphates of the food transformed in greater amount into soluble forms by the ingested acid in the alimentary tract. Würtz (1912) it is true failed to find that feed-

ing hydrochloric acid to rabbits had any effect on their output of P_2O_5 in either urine or feces. The amounts of acid which he fed were, however, only about one-seventh as great as in the experiments of Fitz, Alsberg, and Henderson (1907), and for this reason his negative results are inconclusive. In order to ascertain whether the amounts of acid fed in our experiments influence the distribution of excreted phosphate between feces and urine, we have in some cases determined the excretion in both. The acid feeding showed no definite influence on the distribution. The increased phosphate excretion in the urine was not accompanied by a decrease in the feces, and consequently it appears that the urinary increase in our experiments indicates a genuine negative balance in phosphoric acid—a conclusion which is confirmed by the tissue analyses. These results lend added force to the conclusions of Fitz, Alsberg, and Henderson (1907), that their data indicated a utilization of body phosphates as alkaline reserve to neutralize invading acids and turn them into excretable salts.

EXPERIMENTAL.

I. Effect of Acid Feeding on Phosphate Excretion in Urine and Feces.

As experimental animals, rabbits were chosen in preference to dogs. The ammonia-forming power of dogs enables them to neutralize great amounts of acid without drawing upon the body reserves of mineral alkali. Rabbits can form ammonia much less rapidly (Walter, 1877) and can therefore be expected to neutralize unusual amounts of strong acid only by means of the reserves of alkali in the body fluids, tissues, and skeleton.

The rabbits were kept in metabolism cages and were offered daily 65 gm. of bread and 300 gm. of cabbage. They did not usually consume all that was given, as shown by Table VII. The acid was given in 0.25 N concentration by stomach tube, the amounts varying from 25 to 75 cc. daily. The conditions approximate those of Fitz, Alsberg, and Henderson (1907).

The urine was collected daily at 9 a.m. from Monday to Saturday, and kept in a refrigerator; the urine for the 5 week days was mixed and the total phosphoric acid estimated by titration with uranium nitrate.

Feces were collected daily at 3 p.m. before giving food, and the corresponding amounts of food and feces noted. Feces for 5 days, from Monday to Saturday, were mixed in one dish and ashed. The ash was dissolved by boiling with hydrochloric acid, and the solution was filtered into a 250 cc. volumetric flask and diluted to the mark. Phosphoric acid in 25 cc. portions was precipitated as molybdate and weighed as $Mg_2P_2O_7$.

Results.—The rabbits showed a poor resistance to hydrochloric acid, most of them dying within 1 or 2 weeks. Among the many

TABLE I.

Phosphorus Excretion in Urine of Normal Rabbits.

Date of experiment.		PO_4 in urine per day.			
		Rabbit VIII (body wt. 1,500 gm.).	Rabbit X (body wt. 1,550 gm.).	Rabbit XV (body wt. 1,500 gm.).	Rabbit XVI (body wt. 1,400 gm.).
wk.	*days*	*gm.*	*gm.*	*gm.*	*gm.*
1st	1–6	0.039	0.057	0.037	0.032
2nd	8–13	0.035	0.044	0.066	0.032
3rd	15–20	0.043	0.074 Mixed with feces.	0.017	0.086 Mixed with feces.
4th	22–27	0.033	0.041	0.024	0.020
5th	30–34 (4 days)				0.063
Average		0.038	0.047 (1st, 2nd, 4th weeks.)	0.036	0.037 (1st, 2nd, 4th, 5th weeks.)

rabbits, which were given 50 cc. of 0.25 N HCl daily from the beginning of the experiment, only Rabbit III lived more than 2 weeks. As the object of the experiment was to see the effect of continued acidosis, possibly similar to diabetic acidosis, upon the metabolic changes of the body tissues, it was intended to keep the rabbits at least 4 weeks. Therefore, 25 cc. doses of 0.25 N HCl were given to several of the rabbits. Of these, Rabbit V lived a long time and

TABLE II.

Excretion of PO_4 in Urine of Rabbits Fed with HCl.

Date of experiment.		Acid-fed Rabbit III.			Acid-fed Rabbit IX.			Acid-fed Rabbit XX.			Acid-fed Rabbit XXI.		
		PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.
wk.	*days*	*gm.*	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*	
1st	1–6	0.075	1,500	50 cc. 0.25 N HCl daily.	0.096	1,500	25 cc. 0.25 N HCl daily.	0.110	1,850	30 cc. 0.25 N HCl daily.	0.091	1,400	30 cc. 0.25 N HCl daily.
2nd	8–13	0.135	1,450	50 cc. 0.25 N HCl daily.	0.088	1,400	25 cc. 0.25 N HCl daily.	0.120	1,550	30 cc. 0.25 N HCl daily.			Died 7th day.
			1,310	Died 15th day.			Died 15th day.			Died 15th day.			

TABLE III.

Excretion of PO_4 in Urine of Rabbits Fed with HCl.

Date of experiment.		Acid-fed Rabbit V.			Acid-fed Rabbit XII.			Acid-fed Rabbit XI.			Acid-fed Rabbit VII.				
		PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.	Date of experiment.		PO_4 in urine per day.	Wt. of rabbit.	Remarks.
wk.	*days*	*gm.*	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*		*wk.*	*days*	*gm.*	*gm.*	
1st	1–6	0.122	1,450	25 cc. 0.25 N HCl daily.	0.037	1,650	25 cc. 0.25 N HCl daily.	0.078	1,300	25 cc. 0.25 N HCl daily.	1st	1–3	0.099	1,500	25 cc. 0.25 N HCl daily.
2nd	8–13	0.058	1,300	25 cc. 0.25 N HCl daily.	0.086	1,650	30 cc. 0.25 N HCl daily.	0.051	1,250	30 cc. 0.25 N HCl daily.	2nd	5–10	0.082	1,450	25 cc. 0.25 N HCl daily.
3rd	15–20	0.039	1,250	25 cc. 0.25 N HCl daily.	0.054	1,600	30 cc. 0.25 N HCl daily.	0.036	1,250	30 cc. 0.25 N HCl daily.	3rd	12–17	0.087	1,200	25 cc. 0.25 N HCl daily. Died 17th day.
4th	22–27	0.080	1,250	35 cc. 0.25 N HCl daily.			Died 22nd day.	0.012	1,150	30 cc. 0.25 N HCl daily.					
5th	29–34	0.056	1,250	50 cc. 0.25 N HCl daily.				0.017	1,150	30 cc. 0.25 N HCl daily.					
6th	36–38	0.088	1,250	75 cc. 0.25 N HCl daily. Died 38th day.						Died 35th day.					

received increased doses of acid. Rabbits XI and XII received 30 cc. of 0.25 N HCl from the 2nd week. Rabbits XX and XXI received 30 cc. of 0.25 N HCl from the beginning of the experiment, and lived less than 3 weeks, but were examined to ascertain the effect of the shorter period of acid feeding.

TABLE IV.

Phosphorus in Feces of Normal Rabbits.

Date.		PO_4 per day.	
		Rabbit XV.	Rabbit XVI.
wk.	*days*	*mg.*	*mg.*
3rd	15–20	12.5	38.6
4th	22–27	40.7	87.5
5th	30–34		65.0

TABLE V.

Phosphorus in Feces of Acid-Fed Rabbits.

Date of experiment.		PO_4 per day.			
		Rabbit XI.	Rabbit XII.	Rabbit XX.	Rabbit XXI.
wk.	*days*	*mg.*	*mg.*	*mg.*	*mg.*
1st	1–6			39.6	56.9
2nd	8–13			49.5	
3rd	15–20	120	153		
4th	22–27	112			
5th	30–34	26			

Four normal controls of almost similar weight showed an average daily PO_4 excretion of 0.036 to 0.047 gm. (Table I). Of the experimental rabbits, Nos. III, V, VII, IX, XI, XX, and XXI excreted more phosphate even in the 1st week than the controls (Tables II and III). Rabbit XII did not show the increased excretion in the 1st week, but did show an excessive excretion in the 2nd and 3rd

TABLE VI.

Diet of Normal Rabbits in Which Phosphoric Acid of Feces Was Determined.

Date of experiment.		Rabbit XV.		Rabbit XVI.	
		Bread.	Cabbage.	Bread.	Cabbage.
wk.	*days*	*gm.*	*gm.*	*gm.*	*gm.*
3rd	15–16	65	300	65	300
	16–17	65	285	47	235
	17–18	50	187	52	200
	18–19	27	121	57	270
	19–20	37	130	52	202
4th	22–23	45	243	48	233
	23–24	58	223	58	300
	24–25	47	193	50	300
	25–26	61	220	54	222
	26–27	—	—	—	—
5th	30–31	—	—	58	300
	31–32	—	—	59	300
	32–33	—	—	43	172
	33–34	—	—	46	154

TABLE VII.

Diet of Acid-Fed Rabbits in Which Phosphoric Acid of Feces Was Determined.

Date of experiment.		Rabbit XI.		Rabbit XII.		Date of experiment.		Rabbit XX.		Rabbit XXI.	
		Bread.	Cabbage.	Bread.	Cabbage.			Bread.	Cabbage.	Bread.	Cabbage.
wk.	*days*	*gm.*	*gm.*	*gm.*	*gm.*	*wk.*	*days*	*gm.*	*gm.*	*gm.*	*gm.*
3rd	15–16	65	275	65	300	1st	1–2	—	300	—	300
	16–17	65	229	42	239		2–3	—	300	—	245
	17–18	53	166	56	200		3–4	40	200	—	157
	18–19	48	193	41	268		4–5	54	300	46	126
	19–20	49	148	38	171		5–6	38	300	31	98
4th	22–23	38	281			2nd	8–9	16	226		
	23–24	47	204				9–10	45	293		
	24–25	12	107				10–11	22	178		
	25–26	19	168				11–12	17	133		
	26–27	—	—				12–13	—	—		
5th	30–31	44	171								
	31–32	42	139								
	32–33	31	143								
	33–34	20	135								

weeks. Rabbit XI showed a normal excretion in the 3rd week, followed by diminished excretion in the 4th and 5th weeks. Rabbit V also showed a normal phosphate excretion in the 3rd week, but as he received gradually increased doses of acid after that period, the results are not comparable with those of Rabbit XI. When the amount of ingested hydrochloric acid rose, a corresponding increase of phosphoric acid excretion occurred.

The PO_4 in the feces of the control rabbits was 12.5 to 87.5 mg. per day (Table IV), in the acid-fed rabbits 26 to 153 mg. (Table V). It is evident that the acid feeding caused no decrease in fecal phosphorus, the change being, if anything, in the opposite direction.

II. The Influence of Acid Feeding in the Rabbit on the Bicarbonate Content of the Plasma.

Walter (1877), who made the first experiments in acid intoxication with dogs and rabbits, found great decrease in the carbon dioxide content of the blood. The results of later experimenters agree with Walter's. The effects of HCl administration in the dog upon the CO_2 content of the plasma, estimated by the Van Slyke method, were recently reported by the writer (1917).

25 cc. of 0.25 N HCl were administered by stomach tube to several normal rabbits daily at 2 p.m. Blood was drawn by heart puncture before the acid feeding, and the CO_2 content of the plasma estimated by the Van Slyke method (Table VIII). The rabbits died in about 1 week after the acid administration, since in their weakened condition they could not stand the heart puncture. Bicarbonate CO_2 in the plasma of the controls was 44 to 66 per cent. In the 1st week of the acid administration (3rd day) several rabbits showed a lowered CO_2 in the plasma. These rabbits did not live to the 2nd week. Rabbit XXVIII showed no acidosis in the 1st, but a marked acidosis in the 2nd week. Rabbit XXVII, which was not bled in the 2nd week, but which received acid every day, also showed a marked acidosis in the 3rd week. Both rabbits died after heart puncture. Rabbit XI, used in one of the previous experiments, showed 34.9 per cent CO_2 content in the plasma in the 4th week (April 30).

TABLE VIII.

Carbon Dioxide Content of Plasma.

	Date.	Rabbit XXIV.	Rabbit XXV.	Rabbit XXVI.	Rabbit XXVII.	Rabbit XXVIII.	Rabbit XXIX.	Remarks.
		vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
Normal condition.	May 21	44.0	44.0	44.0	57.2	60.0	53.4	
	" 24	54.4	66.0	63.9	51.5	57.3	63.0	
1st week of acid feeding.	May 29	31.2 Died May 30.	45.6	36.9 Died May 31.	51.1	63.8	43.6 Died May 31.	From May 27. 25 cc. 0.25 N HCl given daily.
2nd week of acid feeding.	June 5		Died June 2.			33.6 Died June 5.		
3rd week of acid feeding.	June 12				31.2 Died June 12.			

III. Mineral Changes in Muscles and Bones.

After the deaths of the rabbits which had been used for the phosphate excretion experiments reported above, the muscles and bones were separated as completely as possible. The total muscles (except the heart) were weighed, ground in a food chopper, weighed again, and kept in a refrigerator. From them phosphorus, potassium, and sodium were determined. The bones from the entire body, including the bone marrow, were weighed, dried on a water bath for 4 or 5 days, ground in a hand mill, weighed again, and kept in a bottle. In the bone powder, phosphoric acid, calcium, carbonic acid, and fat were estimated.

Age effects a change in the mineral constituents of the bones of rabbits (Weiske, 1872, Wildt, 1872), but the changes are minimal in rabbits from 1 to 3 or 4 years of age. Adult animals from 1 to 2 years of about the same weight were selected for the present research.

Methods of Analysis.

Phosphoric Acid in Muscle Ash.—50 gm. of fresh muscle were weighed into a platinum dish and ashed, first over a flame and finally in a muffle furnace, using full red heat only after all organic matter was entirely turned to charcoal. To the residue 15 cc. of water and 3 to 5 cc. of concentrated hydrochloric acid were added, and the ash was dissolved with the aid of heat. The solution was washed into a 200 cc. flask, diluted to the mark, and filtered into an Erlenmeyer flask through a dry folded filter paper to remove particles of carbon. 50 cc. portions of this solution were taken for phosphate determination. The phosphate was precipitated as molybdate and weighed as $Mg_2P_2O_7$.

Potassium and Sodium in Muscle.—After ashing the muscle and dissolving the residue in the same manner, 5 cc. of 10 per cent $BaCl_2$ were added and followed by 10 per cent $Ca(OH)_2$ suspension till the solution was alkaline to litmus. The mixture was diluted to 150 cc. and filtered through a folded filter paper to remove phosphates and sulfates. To 100 cc. of the filtrate 20 cc. of saturated ammonium oxalate were added, followed by 10 per cent ammonium carbonate until no further precipitate was obtainable. The solution was diluted to 200 cc. and filtered through a folded filter paper to remove calcium and barium. 50 cc. of the filtrate were placed in a weighed silica dish with an excess of concentrated hydrochloric acid and evaporated to dryness on a water bath. The residual KCl and NaCl were ignited and weighed. The chlorides were dissolved in a small amount of water, 0.5 cc. of perchloric acid was added, and the solution evaporated to dryness. After addition of 3 or 4 cc. of water and five drops of perchloric acid, the solution was evaporated to dryness again. The residue was stirred up with 10 cc. of 97 per cent alcohol containing 0.2 per cent of perchloric acid, allowed to stand at least 20 minutes, and then transferred to a Gooch crucible, in which it was washed several times with small portions of the same alcoholic solution containing 0.2 per cent of perchloric acid. The $KClO_4$ was dried at 110°C. and weighed.

Phosphoric Acid Determination in Bones.—10 gm. of dried bone powder were ashed in a platinum dish as above. The ash was trans-

ferred to a 200 or 300 cc. beaker, with 50 to 100 cc. of water. The latter was boiled and hydrochloric acid added until all solid matter except carbon was dissolved. The solution was filtered into a 250 cc. flask. The phosphate in 20 cc. was precipitated as molybdate and weighed as $Mg_2P_2O_7$.

Calcium Determination in Bones.—To 10 cc. of the bone ash solution, prepared as above, 5 cc. of 10 per cent sulfuric acid and 60 cc. of 95 per cent alcohol were added. The solution was let stand over night. The $CaSO_4$ precipitate was filtered into an alundum crucible, washed with 70 per cent alcohol, and dried at 100°. The alundum crucible was then set inside a porcelain one, which was heated to a dull red for about 30 minutes.

Carbonic Acid Determination in Bones.—Van Slyke's new carbonate method was used (1918), as described in the preceding article. 1 gm. samples were used of the dried bone powder.

Determination of Fat in Bone.—1 gm. of dried bone powder was extracted in a Soxhlet extractor with 150 cc. of absolute ether for 24 hours. The ether was driven off on a water bath. 50 cc. of petroleum ether were added and let stand over night on the residue. The solution was filtered through fat-free cotton, the petroleum ether drawn off, and the residue weighed as fat.

Results.

I. Muscles.

1. Weight of Muscles.—The weight of the total muscles per kilo of body weight in four control rabbits was practically constant, varying only from 318 to 355 gm. (Table IX). In the experimental rabbits, the ratio of weight of muscles to body weight was calculated on the basis both of final body weight and of body weight before acid feeding was begun, since the animals lost weight during the period of acid feeding. The weight of muscles per kilo of initial body weight, except for Rabbits XII and XXI, was lower in the acid-fed rabbits than in any of the controls, indicating a loss of muscle tissue during acid feeding (Table X).

The weight of muscles per kilo of final body weight was lower than normal in Rabbits V, VII, IX, XI, and XX which had received a

long continued acid feeding (13 to 38 days). The ratio in Rabbits XXI and XII was almost normal, in spite of the fact that they had received acid for 2 weeks or more. These results show that such loss of weight as occurred fell more heavily on the muscles than on the body as a whole.

TABLE IX.

Weight of Muscles in Normal Rabbits.

Rabbit No.	Body wt.	Weight of muscles.		
		Before maceration.	After maceration.	
				Per kg. body wt.
	gm.	*gm.*	*gm.*	*gm.*
VIII	1,500	530.6	493.3	355
X	1,500	521.6	477.1	318
XV	1,500	544.0	523.8	350
XVI	1,400	476.8	449.0	321
Average				336

TABLE X.

Weight of Muscles in Acid-Fed Rabbits.

Rabbit No.	Duration of experiment.	Body weight.		Weight of muscles.		Weight of muscles.	
		Initial.	Final.	Before maceration.	After maceration.	Per kg. initial body wt.	Per kg. final body wt.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
III	15	1,500	1,310	430.5	415.0	277	320
V	38	1,450	1,250	335.2	319.5	220	255
VII	17	1,500	1,200	347.1	331.8	222	277
IX	13	1,500	1,400	442.1	424.0	283	303
XI	35	1,240	1,150	272.0	262.8	212	229
XII	22	1,650	1,600	579.0	561.0	340	350
XX	14	1,850	1,500	486.0	472.0	255	314
XXI	7	1,400	1,340	482.0	463.0	331	346

2. Change in Phosphorus Content of Muscles.—The control rabbits showed almost constant results, the percentage of PO_4 in the fresh muscles being 0.759 to 0.883 and the total PO_4 per kilo of body weight being 2.6 to 2.8 gm. The percentage of PO_4 in the muscles of acid-fed rabbits was in some cases normal, but in four

of the eight animals it was lower than the lowest normal. The ratio of total PO_4 per kilo of initial body weight was remarkably lower than normal in all the rabbits which had received acid except Rabbit III. From the results it is evident that the total phosphorus in the muscles of rabbits is reduced by acid feeding, and that on the average, the reduction includes the percentage of phosphorus in the muscles as well as the amount in the total musculature.

3. Changes in Sodium Content of the Muscles.—The sodium content in the muscles of the normal animals showed an average of 0.0973 per cent, the extremes being 0.078 to 0.122. The total sodium per kilo of body weight was 0.25 to 0.39 gm., the average being 0.32 gm. for the four controls. A moderately high percentage of sodium

TABLE XI.

Analyses of Muscles of Normal Rabbits.

Rabbit No.	PO_4			Na			K		
	PO_4 in muscles.	Total PO_4 in all muscles.	Total PO_4 per kilo body wt.	Na in muscles.	Total Na in all muscles.	Total Na per kilo body wt.	K in muscles.	Total K in all muscles.	Total K per kilo body wt.
	per cent	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*
VIII	0.777	3.84	2.6	0.0988	0.487	0.33	0.301	1.49	0.99
X	0.883	4.21	2.8	0.122	0.582	0.39	0.252	1.20	0.80
XV	0.759	3.97	2.7	0.0903	0.473	0.32	0.32	1.68	1.12
XVI	0.796	3.58	2.6	0.078	0.351	0.25	0.385	1.73	1.24
Average.	0.804		2.64	0.0973		0.32	0.315		1.04

is seen in the acid-fed rabbits, Nos. III, VII, and XXI. The ratio of the total sodium, both per kilo of initial body weight and per kilo of final body weight was lower than in the lowest control in Rabbits V, XI, and XX, which had received long courses of acid feeding.

4. Changes in Potassium Content of the Muscles.—In the normal rabbits the percentage of potassium in the muscles was 0.252 to 0.385, and the total potassium per kilo of body weight was 0.8 to 1.24 gm., the average being 1.04 gm. The percentage of potassium in the muscles of acid-fed rabbits, Nos. V, XI, XII, and XXI, was lower than the lowest normal. The ratio of total potassium per kilo of

TABLE XII.

Analyses of Muscles of Acid-Fed Rabbits

Rabbit No.	Duration of experiment.	No. of days of acid feeding.	Total volume of 0.25 N HCl fed.	PO_4				Na				K			
				PO_4 in muscles.	Total PO_4 in all muscles.	Total PO_4 in muscles.		Na in muscles.	Total Na in all muscles.	Total Na in muscles.		K in muscles	Total K in all muscles.	Total K in muscles.	
						Per kg. initial body wt.	Per kg. final body wt.			Per kg. initial body wt.	Per kg. final body wt.			Per kg. initial body wt.	Per kg. final body wt.
	days		*cc.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*
V	38	33	1,185	0.752	2.41	1.7	1.9	0.086	0.275	0.19	0.22	0.163	0.52	0.36	0.42
XI	35	29	810	0.827	2.17	1.8	1.9	0.084	0.221	0.18	0.19	0.251	0.66	0.58	0.59
III	15	12	600	0.933	3.87	2.6	2.95	0.132	0.584	0.37	0.42	0.288	1.195	0.80	0.91
XII	22	18	510	0.689	3.86	2.4	2.4	0.087	0.488	0.30	0.31	0.219	1.23	0.75	0.77
VII	17	15	375	0.595	1.99	1.3	1.7	0.141	0.467	0.31	0.39	0.268	0.896	0.59	0.74
XX	14	12	360	0.849	4.01	2.2	2.7	0.067	0.316	0.17	0.21	0.302	1.435	0.77	0.95
IX	13	12	300	0.733	3.11	2.1	2.2	0.0973	0.413	0.28	0.30	0.254	1.08	0.72	0.77
XXI	7	6	180	0.604	2.80	2.0	2.1	0.132	0.611	0.44	0.46	0.222	1.03	0.74	0.77

initial body weight of all the acid-fed rabbits except No. III was lower than the lowest normal. The ratio of total potassium per kilo of final body weight in all the experimental animals, except Nos. III and XX, was lower than normal. It appears that potassium is considerably decreased in the muscles by experimental acidosis.

II. Bones.

1. Changes in Weight of Dried Skeleton.—The weight of dried bone per kilo of body weight was 54 to 65 gm. in the normal rabbits and averaged 59 gm. (Table XIII). The weight of bone per kilo of initial body weight was lowered to 40 to 54 gm. in the acid-fed rabbits averaging 50 gm. (Table XIV). The ratio of the weight of bone per kilo of final body weight was normal in the acid-fed rab-

TABLE XIII.

Weight of Skeleton in Normal Rabbits.

Rabbit No.	Wet.	After drying.	After crushing.	Per kg. body wt.
	gm.	*gm.*	*gm.*	*gm.*
VIII	140.5	86.2	80.8	54
X	158.9	94.4	83.0	55
XV	169.0	99.4	97.4	65
XVI	130.5	87.6	87.4	62
Average				59

bits, except in Rabbit V which had endured a long continued acid feeding. These results indicate that the dry weight of the skeleton decreases parallel with the weight of the total body during acid feeding. As shown below, about half the bone loss is attributable to decrease in the bone fat.

2. Changes in Skeletal Phosphate.—The PO_4 percentage in the dried bones of normal rabbits was 20.8 to 23.3 and the total PO_4 per kilo of body weight was 11.2 to 14.3 gm.

All of the acid-fed rabbits showed a percentage of PO_4 in the bones rather higher than normal because of a decreased fat content, rather than an increase of phosphate, as will be indicated later. The ratio of the total PO_4 per kilo of initial body weight was lower than nor-

mal only in Rabbit V of the acid-fed rabbits. Rabbit V showed a smaller amount of all bone substances. Whether this was due to the exceptional amount of acid given or to a chance individual variation

TABLE XIV.

Weight of Skeleton in Acid-Fed Rabbits.

Rabbit No.	Duration of experiment.	Weight of bone.				
		Wet.	After drying.	After crushing.	Per kg.	
					Initial body wt.	Final body wt.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
III	15	109		71.8	48	55
V	38	119	61	58.5	40	47
VII	17	134		68.5	46	57
IX	13	148		81.1	54	58
XI	35	129	65.2	65.2	53	57
XII	22	166	90.4	88.4	54	55
XX	14	158	95	93.6	51	62
XXI	7	158	75.6	72.4	52	54

TABLE XV.

Analyses of Bones of Normal Rabbits.

Rabbit No.	PO_4			Ca			CO_2			Fat.		
	PO_4 in dry bone.	PO_4 in total skeleton.	Total PO_4 per kg. body wt.	Ca in dry bone.	Ca in total skeleton.	Total Ca per kg. body wt.	CO_2 in dry bone.	CO_2 in total skeleton.	Total CO_2 per kg. body wt.	Fat in dry bone.	Fat in total skeleton.	Total fat per kg. body wt.
	per cent	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*
VIII	20.8	16.8	11.2	13.1	10.6	7.6	5.1	4.1	2.8	19.59	15.8	10.5
X	21.1	17.5	11.7	14.1	11.7	7.8	5.2	4.3	2.9	14.5	12.0	8.0
XV	21.7	21.0	14.0	14.7	14.3	9.5	4.8	4.7	3.1	18.21	17.7	11.8
XVI	23.3	20.3	14.3	15.2	13.3	9.5	4.9	4.3	3.1	16.22	14.1	10.1
Average...	21.7		12.8	14.3		8.6	5.0		2.95	17.13		10.1

from the normal, one cannot say. The bones of the other rabbits indicate no significant effect of the acid feeding on the phosphate content of the skeleton.

TABLE XVI.

Analyses of Bones of Acid-Fed Rabbits.

Rabbit No.	Duration of experiment.	No. of days of acid feeding.	Total volume of 0.25 N HCl fed.	PO_4				Ca				CO_2				Fat.			
				PO_4 in dry bone.	PO_4 in total skeleton.	PO_4 per kg. initial body wt.	PO_4 per kg. final body wt.	Ca in dry bone.	Ca in total skeleton.	Ca per kg. initial body wt.	Ca per kg. final body wt.	CO_2 content of dry bone.	CO_2 in total skeleton.	CO_2 per kg. initial body wt.	CO_2 per kg. final body wt.	Fat in dry bone.	Fat in total skeleton.	Bone fat per kg. initial body wt.	Bone fat per kg. final body wt.
	days		*cc.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*
V	38	33	1,185	24.2	14.2	9.8	11.3	15.25	8.9	6.2	7.1	5.1	2.98	2.1	2.4	8.97	5.3	3.6	4.2
XI	35	29	810	25.7	16.7	13.5	14.6	17.1	11.2	9.0	9.7	4.7	3.06	2.5	2.7	3.53	2.3	1.9	2.0
III	15	12	600	24.9	17.9	11.9	13.6	16.8	12.1	8.0	9.2	5.1	3.66	2.4	2.8	9.88	7.1	4.7	5.4
XII	22	18	510	21.4	19.8	12.0	12.4	14.1	12.5	7.6	7.8	4.6	4.07	2.5	2.5	14.03	12.4	7.5	7.8
VII	17	15	375	27.2	18.6	12.4	15.5	16.85	11.6	7.7	9.7	5.0	3.42	2.3	2.9	7.02	4.8	3.2	4.0
XX	14	12	360	25.8	24.1	13.1	16.0	17.9	16.8	9.1	11.2	5.6	5.27	2.8	3.5	8.43	6.95	3.8	4.6
IX	13	12	300	25.1	20.3	13.6	14.5	16.25	13.2	8.8	9.4	5.2	4.20	2.8	3.0	6.94	5.6	3.6	4.0
XXI	7	6	180	22.8	16.5	11.8	12.3	13.8	10.0	7.1	7.5	4.7	3.26	2.3	2.4	9.99	7.2	5.2	5.4

3. Changes in Skeletal Calcium.—The percentage of calcium in the bones of the normal rabbits varied from 13.1 to 15.2, and the total calcium per kilo of body weight from 7.6 to 9.5 gm., the average being 8.6 gm. The acid-fed rabbits showed 13.8 to 17.9 per cent of calcium, and 6.2 to 9.1 gm., the average being 7.9 gm., of skeletal calcium per kilo of initial body weight. The last mentioned figures indicate that there was probably some loss of calcium during acid feeding.

4. Changes in Skeletal Carbonate.—The percentage of carbon dioxide in the bones of normal rabbits was 4.8 to 5.2; in the bones of acid-fed rabbits it was 4.6 to 5.6. The total CO_2 per kilo of body weight was 2.8 to 3.1 gm. in the control rabbits; 2.1 to 2.8 in the acid-fed. The ratio of the total carbon dioxide per kilo of initial body weight of the acid-fed rabbits was lower than the lowest normal in six of the eight acid-fed rabbits From these results it appears that the calcium carbonate of the bones, unlike the phosphate, is reduced by acid feeding. This conclusion is in agreement with that reached by Steenbock, Nelson, and Hart (1914) as the result of metabolism experiments on a calf. The fact that the *percentage* of CO_2 is not reduced in bones of acid-fed rabbits is attributable to the loss of fat which makes the relative proportions of other constituents appear higher.

5. Changes in Bone Fat.—The percentage of fat in the bones of the normal animals was 14.5 to 19.6 averaging 17.1, and the total fat per kilo of body weight was 8.0 to 11.8 gm. In the acid-fed rabbits, the fat percentage was 3.5 to 14.0 averaging 8.6 or half as high as in controls. The ratio of the total fat per kilo of initial body weight was correspondingly reduced. The reduction occurs even in the early stages of experimental acidosis. Because of this reduction in bone fat, the figures for the percentages of PO_4 and Ca in the bones of acid-fed rabbits are rather higher than normal. The bone powder of the acid-fed rabbits was dryer and visibly less fatty than that of normal rabbits, and it was this appearance which led us to determine the relative fat content.

SUMMARY.

Daily administration of 25 to 75 cc. of 0.25 N hydrochloric acid to rabbits for 1 to 4 weeks had the following effects:

1. Blood plasma bicarbonate was reduced.

2. Phosphoric acid excretion was increased, the increase being in some cases followed by a fall. These observations confirm those of Fitz, Alsberg, and Henderson (1907).

3. Phosphate excretion in the feces was not markedly affected.

4. The muscles lost markedly in phosphorus and potassium and somewhat in sodium.

5. In the skeleton the chief effect was a great reduction in fat content, which fell from an average of 17.1 per cent of the dry weight to 8.6 per cent. Aside from the fat loss, the skeletons of acid-fed rabbits averaged about 10 per cent lighter in dry weight than those of the normals. There was no demonstrable loss of calcium phosphate. There was a definite loss on the average of about one-fifth of the carbon dioxide, indicating that the calcium carbonate is more readily sacrificed in acid intoxication than is the phosphate.

CONCLUSIONS.

The individual variations in muscle and bone are such, that in every constituent determined, the figures for some of the acid-fed rabbits overlap the normal range. Consequently the interpretation of results is not so sharp as could be desired, and it is regrettable that the time available has not permitted the extension of the experiments to a larger number of animals, so that the data could be put on a statistical basis.

The results nevertheless indicate with a fair degree of decisiveness that next to the bicarbonates of the body fluids, the first major reserves of alkali drawn upon in acid intoxication of rabbits are the alkali phosphates, particularly potassium phosphate, of the muscles, and the calcium carbonate of the bones. The bones do not lose calcium phosphate in appreciable amounts.

The above results are only suggestive in their relationship to clinical acidosis. Whether a loss of alkali phosphates from the tissues and calcium carbonate from the bones occurs in the latter remains to be ascertained by accurate determination of mineral balances.

The writer's thanks are due to Dr. Donald D. Van Slyke for his constant advice during the course of this work.

BIBLIOGRAPHY.

Fitz, R., Alsberg, C. L., and Henderson, L. J., *Am. J. Physiol.*, 1907, xviii, 113.
Folin, O., and Shaffer, P. A., *Am. J. Physiol.*, 1902, vii, 135.
Forbes, E. B., and Keith, M. H., A review of the literature of phosphorus compounds in animal metabolism, *Ohio Agric. Exp. Sta., Technical Bull. 5*, 1914.
Gerhardt, D., and Schlesinger, W., *Arch. exp. Path. u. Pharmakol.*, 1899, xlii, 83.
Givens, M. H., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 421.
Givens, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1918, xv, 66.
Goto, K., *J. Exp. Med.*, 1917, xxv, 693.
Mandel, A. R., and Lusk, G., *Deutsch. Arch. klin. Med.*, 1904, lxxxi, 472.
Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.
Rumpf, T., *Berl. klin. Woch.*, 1898, xxxv, 945.
Steenbock, H., Nelson, V. E., and Hart, E. B., *J. Biol. Chem.*, 1914, xix, 399.
Stehle, R. L., *J. Biol. Chem.*, 1917, xxxi, 461.
Tenbaum, E., *Z. Biol.*, 1896, xxxiii, 379.
Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxvi, 351.
Walter, F., *Arch. exp. Path. u. Pharmakol.*, 1877, vii, 148.
Weiske-Proskau, H., *Z. Biol.*, 1872, viii, 239.
Wildt, E., *Landw. Versuchssta.*, 1872, xv, 404, cited by Forbes and Keith.
Würtz, A., *Biochem. Z.*, 1912, xlvi, 103.

[Reprinted from The Journal of Experimental Medicine, September 1, 1918,
Vol. xxviii, No. 3, pp. 253–267.]

STUDIES IN BOVINE MASTITIS.

II. The Relation of Hemolytic Streptococci to Udder Infections.

By F. S. JONES, V.M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, May 28, 1918.)

INTRODUCTION.

Epidemics of milk-borne tonsillitis have aroused considerable interest in the study of bovine streptococci, especially those responsible for disorders of the mammary gland. In a number of instances streptococci isolated directly from the udders of cows have been identical with those found in the throats of patients suffering from tonsillitis. The question having arisen as to the source of these organisms, the general tendency is to consider them human streptococci which have gained access to the udder of the cow through the milk duct. If this is true, they are able to live and multiply within the udder for a certain period. There still exists uncertainty as to their power to produce clinical mastitis beyond alterations in the character of the milk secreted from the infected quarters.

Savage[1] in his descriptions of streptococci obtained from the milk of cows suffering from mastitis did not consider their behavior on blood agar plates. The hemolytic activity of certain streptococci isolated from inflamed udders, mixed herd milk, and market milk has been reported by a few authors. Smith and Brown[2] in their studies of certain milk-borne epidemics of tonsillitis describe ten strains of bovine hemolytic streptococci which they isolated from milk drawn directly from the udder. Davis[3] studied a number of hemolytic streptococci ob-

[1] Savage, W. G., *Rep. Med. Off. Local Gov. Bd. 1906–07*, xxxvi, 253; *Rep. Med. Off. Local Gov. Bd. 1907–08*, xxxvii, 359, 425; *Rep. Med. Off. Local Gov. Bd. 1908–09*, xxxviii, p. xxxiii.

[2] Smith, T., and Brown, J. H., *J. Med. Research*, 1914–15, xxxi, 455.

[3] Davis. D. J., *J. Infect. Dis.*, 1916, xix, 236.

tained from market milk and a single strain isolated from the milk of a cow suffering with mastitis.

I am not aware of detailed descriptions of any considerable number of hemolytic streptococci isolated from milk obtained directly from inflamed udders. In a previous paper[4] a certain number of strains of non-hemolytic streptococci obtained from cows suffering from mastitis were described. At that time it was shown that such organisms were responsible for inflammations of the mammary gland in many cases. When their action upon carbohydrates was considered they fell into two broad groups: those producing acid in dextrose, lactose, saccharose, maltose, and salicin; and those producing acid in dextrose, lactose, saccharose, and maltose, but failing to change the reaction of broth containing salicin. All failed to act upon raffinose, inulin, and mannite. All strains were agglutinated at varying dilutions with an antiserum produced by the immunization of a cow with one strain. Although these streptococci were capable of producing exceedingly severe udder infections in cows, they were not pathogenic when injected into rabbits. A pig fed on milk containing enormous numbers of streptococci remained well.

Of the 61 cases of mastitis associated with streptococci, 21 were associated with hemolytic streptococci, and 38 with non-hemolytic streptococci. In two instances both types were present. In the material at my disposal the non-hemolytic infections predominated, but infections with the hemolytic types existed in considerable number and in all probability they are responsible for serious losses in milk production.

Study of Spontaneous Cases.

The following case records are more or less typical of this class of infections.

Cow 63.—Calved Dec. 8, 1916. Developed mastitis on Apr. 5, 1917.

Apr. 5. The cow refused its food. Digestive disturbances and constipation were observed. The temperature was 103. 6°F. The left fore quarter was enlarged, firm, hot, and tender. The milk was yellowish white in color and thickened with many small viscid flocculi. A sample obtained from the mixed milk of the left and right fore quarters when plated in blood agar revealed 640 hemolytic streptococci per cubic centimeter. The cells numbered 3,200,000 per cubic centimeter.

---

[4] Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 149.

Apr. 10.

Left fore quarter:

The quarter was more flaccid and less tender to manipulation. The appearance of the milk had not changed appreciably.

Cells 6,300,000 per cubic centimeter.

Organisms 76,000 " " " pure culture of streptococci. The surface colonies were round, slightly raised, and translucent. They measured 1 to 2 mm. in diameter. The deep colonies were ovoid. They were surrounded by a zone of complete hemolysis 1 mm. in diameter.

Right fore quarter:

Cells 325,000 per cubic centimeter.

Organisms 20 " " " no hemolytic streptococci.

Left hind quarter:

Cells 295,000 per cubic centimeter.

Organisms 0 " " "

Right hind quarter:

Cells 115,000 per cubic centimeter.

Organisms 0 " " "

Apr. 12.

Left fore quarter:

Cells 6,300,000 per cubic centimeter.

Organisms 0 " " " plates containing 1 cc. of milk remained sterile after incubation.

The quarter appeared normal. The milk still contained flocculi.

Left hind quarter:

Cells 370,000 per cubic centimeter.

Organisms 4,400 " " " pure culture of hemolytic streptococci identical with those obtained from the left fore quarter on Apr. 10.

The quarter appeared normal. A few very small white flakes were observed in the milk.

Right fore quarter:

Cells 170,000 per cubic centimeter.

Organisms 0 " " " plate culture containing 0.5 cc. of milk remained sterile after incubation.

Right hind quarter:

Cells 115,000 per cubic centimeter.

Organisms 0 " " "

This cow was under observation for 2 months. The left fore quarter improved rapidly although the milk secretion never reached normal. Flocculi completely disappeared from the milk. Hemolytic streptococci were never found in the milk after Apr. 12. On Apr. 20, 30 cc. of milk were drawn from the quarter with

a sterile milk tube directly into a sterile bottle. The milk was incubated for 24 hours at 38°C. Tubes containing slant agar and bouillon were inoculated with the incubated milk. They remained sterile. After it had been shown that the milk was sterile it was inoculated with a pure culture of streptococci previously obtained from the quarter. They grew abundantly. The milk from the left hind quarter continued to contain hemolytic streptococci in pure culture for the 2 months allotted to this observation. Mastitis never developed in this quarter.

Cow 59.—Cow calved July 21, 1917. Mastitis developed almost at once.

July 22. The left hind quarter was swollen, firm, and hot. The subcutaneous blood vessels were engorged. The milk was yellowish white in color and contained many small white flocculi. Examination of the milk revealed 37,000,000 cells and 6,000 hemolytic streptococci per cubic centimeter. The surface colonies on blood agar plates measured 1 mm. in diameter. They were round, translucent, and slightly raised above the surface of the medium. The deep colonies were biconvex and surrounded by a zone of complete hemolysis 3 to 3.5 mm. in diameter.

July 26.
Left hind quarter:
Cells 425,000 per cubic centimeter.
Organisms 800 " " " no hemolytic streptococci.
The milk was normal except for a few small flocculi. The inflammation in the quarter had subsided.

The cell counts in samples of milk from the other quarters were well within normal limits. Plate cultures did not reveal hemolytic streptococci.

One frequently observes invasion of one or more quarters with hemolytic streptococci without evidences of inflammation other than a slight increase in the number of cells and the appearance of small flocculi in the milk. The record of Cow 67 is typical of infections of this type. This cow was under observation for 2 months.

Cow 67.—Sept. 5, 1917. Many small flocculi appeared in the milk of the right fore quarter.

A sample was obtained on Sept. 6, at which time the quarter appeared normal. Plates prepared from the milk revealed 2,000 hemolytic streptococci per cubic centimeter. The deep colonies were surrounded with a clear zone of hemolysis 2 to 2.5 mm. in diameter.

Sept. 12.
Right fore quarter:
The quarter appeared normal. The milk contained many flocculi.
Cells 12,600,000 per cubic centimeter.
Organisms 4,800 " " " pure culture of hemolytic streptococci; deep colonies surrounded by a hemolytic zone 2 to 2.5 mm. in diameter.

Left fore quarter:
Cells 750,000 per cubic centimeter.
Organisms 5,500 " " " pure culture of non-hemolytic streptococci.
Right hind quarter:
The milk contained a few small flocculi.
Cells 2,200,000 per cubic centimeter.
Organisms 16,000 " " " pure culture of hemolytic streptococci, surrounded by a zone of hemolysis 1 to 1.25 mm. in diameter.
Left hind quarter:
The milk was normal in appearance.
Cells 4,100,000 per cubic centimeter.
Organisms 690 " " " 95 per cent of colonies were hemolytic streptococci surrounded by a hemolytic zone 3 mm. in diameter.

Severe mastitis developed in the left fore quarter which had been invaded with non-hemolytic streptococci. The condition of the other quarters remained unchanged. Flocculi appeared in all samples of milk sooner or later and persisted throughout. The number of streptococci showed a tendency to increase in all quarters.

This cow aborted early in pregnancy and was slaughtered Jan. 25, 1918. The udder was obtained for histological study. Gross changes of the usual character were observed in the left fore quarter which had been invaded with non-hemolytic streptococci. The other quarters appeared normal except for a slight reddening of the epithelium.

Histological examination of fixed and stained tissues from each quarter revealed strikingly different lesions. Many of the acini of the left fore quarter had become necrotic. In other lobules the epithelium revealed severe degenerative changes. Great masses of leukocytes frequently filled the lumen of the acini and large milk ducts. There was in addition considerable connective tissue proliferation.

In the other portions of the udder where the hemolytic types had gained a foothold the lesions were not so severe. In one or two instances local degenerative changes had occurred in the secreting portion of the quarter. The epithelium of two or three acini in a few lobules had degenerated, and densely packed masses of leukocytes occupied the lumen. In the main the lesions were confined to the larger lactiferous ducts. The lining epithelium was covered with a thin, pink-staining, slightly granular exudate in which a few leukocytes, round cells, and red blood corpuscles had become enmeshed. The epithelium was granular in appearance. The individual cells contained fat droplets. Desquamation had occurred. The subepithelial tissues contained many leukocytes.

Cow 72.—This cow presented a more severe type of infection. The first bacteriological examination made on Dec. 27, 1917 revealed 218,000 streptococci per cubic centimeter of milk. The left hind quarter was swollen, firm, hot, and painful. The condition became chronic and the animal was killed Feb. 12, 1918, and the udder obtained for examination.

The left hind quarter was larger than the others and quite firm. On section the epithelium was brownish pink in color and dry and granular in appearance. The lobules were clearly demarcated. The lining of the milk ducts was reddened and inflamed. The milk cystern and ducts contained considerable greenish white purulent material. The blood vessels were congested throughout the quarter. The other quarters failed to show gross changes.

Examination of sections prepared from pieces of the left hind quarter fixed in Zenker's fluid and stained with methylene blue and eosin revealed severe lesions. In many lobules the epithelium of the acini had been entirely destroyed. All that remained of the individual acini was the connective tissue framework surrounding a few necrotic epithelial cells and dense masses of degenerated leukocytes and fat cells. In other lobules the lesions were not so severe although the nuclei were pyknotic and leukocytes filled the lumen. Certain of the larger ducts were severely involved. The lining cells were necrotic. The passage had become completely occluded with leukocytes and fibrin.

Hemolytic Streptococci Isolated from Inflamed Udders.

The initial cultures were made from liberal samples of milk drawn directly from the udder into sterile wide mouthed bottles. The diluted milk was added to 12 cc. of melted 2 per cent veal bouillon agar and after mixing was poured into a Petri dish containing 1 cc. of defibrinated horse blood. The dish was agitated quickly to insure a mixture of the contents. Hemolytic readings were made from the deep colonies after incubation for 24 hours. Although the number of strains studied has been somewhat limited, it is felt that detailed descriptions relative to their source, the extent of disease produced, and the morphological and biological characters may be of value. All streptococci isolated directly from the udder have produced in horse blood agar plate cultures a definite clear zone of hemolysis immediately surrounding the colonies which Smith and Brown have described as hemolysis of the beta type.

Table I denotes the number of the streptococcus, the zone of hemolysis, the number of colonies developing from 1 cc. of freshly drawn milk, the appearance of the secretion, and a note on the clinical condition of the involved quarter.

Of the twenty-nine strains isolated, twenty produced more or less pronounced changes in the invaded quarters. In nine instances the freshly drawn milk contained hemolytic streptococci often in considerable numbers, although changes in the udder could not be detected.

Two cows while affected with mastitis in certain quarters shed streptococci from other apparently normal quarters.

Table II denotes the morphological and biological characters of the strains of hemolytic streptococci isolated from milk drawn from the udder. Fermented veal bouillon containing 1 per cent of the various carbohydrates and other substances was used to test the fermentative action of each strain. The cultures were incubated 5 days at 38°C. The initial reaction of the media varied between 0.6 and 0.8 per cent of a normal solution of acid (phenolphthalein). Tubes 1.5 cm. in diameter containing 13 cc. of media were always employed. Only the net production of acid is recorded in the table.

Two distinct types of surface colonies have been noted in blood agar plate cultures. The most frequent appeared as round, flattened, almost transparent colonies measuring from 2 to 4 mm. in diameter. The other type was smaller, raised, and translucent. Deep colonies were usually biconvex or ovoid in shape.

Like the non-hemolytic streptococci, the hemolytic varied greatly in the length of chains produced. The individual elements were spherical or slightly elongated; all stained by Gram's method. Some grew throughout the bouillon producing more or less turbidity; others grew as large flocculi on the bottom of the tube leaving the medium clear. All strains coagulated milk to a greater or less degree. In one instance the milk appeared unchanged after incubation for 5 days. Boiling, however, produced prompt clotting. The hemolytic streptococci fall into the same broad groups as the non-hemolytic strains, when their action upon the carbohydrates is considered. All produced acidity in dextrose, lactose, saccharose, and maltose. Nineteen strains fermented salicin; the other ten failed to do so. One of the non-salicin-fermenting types failed to produce acidity in saccharose. This culture produced comparatively little acid in dextrose, lactose, and maltose. All failed to ferment raffinose, inulin, and mannite. In the main about the same amount of acid production is observed in the sugars fermented by each group.

Smith and Brown's streptococci from bovine sources possessed similar characters. In their series the non-salicin-fermenting strains predominated. The acid production of their streptococci corresponds in a great measure with the results given in Table II.

TABLE I.

Strain No.	Diameter of total area of hemolysis.	No. of streptococci per cc. of milk.	Appearance of milk.	Clinical condition of the quarter.
	mm.			
C.53	1	76,000; pure culture.	Thickened, yellow; contained many irregular flocculi.	Swollen, firm, hot, and tender.
C.53L.H.Q.	1	4,400 " "	Apparently normal.	Apparently normal.
C.57	1		Yellow and flocculent.	
C.59	3–5	6,000; pure culture.	" contained a few fine flocculi.	Enlarged, firm, and corded.
C.63A.	2	36,000 " "	Normal except for a few small white flocculi.	Apparently normal.
C.63B.	0.75	12,000 " "	" " " " " " " "	" "
C.65B.	2.5–3		*	Typical lesions of mastitis.
C.67A.	2.5–3	78,000; pure culture.	Normal except for a few small white flocculi.	A trifle firm.
C.67B.	1	64,500 " "	" " " " " " " "	Firmness of lower third.
C.67C.	3	16,600 " "	" " " " " " " "	Apparently normal.
C.67E.	0.75	184,000	" " " " " " " "	" "
C.69	2–2.5	660; pure culture.	Yellow, thickened with many large, yellow flocculi.	Swollen, hot, and tender.
C.72	1	218,000 " "	Yellow, watery; contained many fine flocculi.	" " firm, and tender.
M.B.	1	33,000 " "	Watery; contained many small flocculi.	Shrunken and firm.
M.C.	1	230,000,000 " "	Thickened, purulent, tinged with green.	Swollen and very firm.
M.R.	3.5–4	306,000	" contained many yellow flocculi.	" firm, hot, and tender.
M.26R.H.	2	4,200; 95 per cent hemolytic streptococci.	Watery; contained many tiny flocculi.	Normal except for a partial occlusion of the teat canal.
M.26L.H.	2	1,710; 15 per cent colonies of hemolytic streptococci.	Apparently normal.	Apparently normal.

M.26R.F.	2	230; 65 per cent colonies of hemolytic streptococci.	Apparently normal.	Apparently normal.
M.29	1	780; pure culture.	Normal except for moderate number of tiny white flocculi.	Firm and hot.
M.43	3–3.5	84,000 " "	Watery; contained a few irregular white flocculi.	Apparently normal.
M.47	1.5	170 " "	Normal, but contained many small, white flocculi.	Irregular, firm, swelling surrounding the cystern.
M.57	Very narrow.	38,000 " "	Yellow, watery; contained many flocculi.	Swollen and firm.
M.59	1.5–2	8,000 " "	Contained a few irregular white "	Small, firm nodule about the cystern.
M.60	Very narrow.	200 " "	Yellow; contained many irregular "	
M.74	0.75	20,000 " "	" purulent, and flocculent.	Swollen, firm, hot, and tender.
M.84	0.75	2,200 " "	Contained many small white flocculi.	Thickening of teat canal.
M.85	3	18,000 " "	Yellow, watery; contained many flocculi.	Apparently normal.
M.89	1	2,900 " "	" " " " "	Swollen, firm, irregular mass 5 cm. in diameter about the cystern.

* The udder was obtained from the abattoir. The milk in the cystern and large ducts was yellow, purulent, and flocculent. Cultures from the milk revealed both hemolytic and non-hemolytic streptococci.

TABLE II.

Morphological and Biological Characters of Hemolytic Streptococci from Inflamed Udders.

Strain No.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C.63	L.C.*	+	Clear.	Partially coagulated.	4.4	4.0	4.2	3.7	0.0	0.0	0.0	2.8
C.63L.H.Q.	"	+	"	" "	4.3	4.0	4.1	3.6	0.0	0.0	0.0	2.4
C.57	"	+	"	Firmly "	4.0	3.5	3.7	3.8	0.0	0.0	0.0	0.0
C.59	M.C.	+	"	" "	4.1	3.9	3.6	3.7	0.0	0.0	0.0	3.0
C.63A.	L.C.	+	Turbid.	" "	4.6	3.7	3.6	4.0	0.0	0.0	0.0	2.9
C.63B.	S.C.	+	"	" "	4.2	3.5	3.3	3.3	0.1	0.1	0.0	1.5
C.65B.	L.C.	+	"	Coagulated on boiling.	3.7	3.2	3.0	3.3	0.0	0.0	0.0	2.6
C.67A.	"	+	Clear.	Firmly coagulated.	5.0	3.9	3.8	3.8	0.1	0.1	0.0	3.3
C.67B.	M.C.	+	Turbid.	" "	5.2	4.5	4.2	4.0	0.0	0.1	0.1	0.1
C.67C.	"	+	"	" "	5.4	4.5	4.2	4.4	0.0	0.0	0.0	0.0
C.67E.	S.C.	+	"	" "	4.8	3.3	3.4	3.1	0.1	0.1	0.0	1.3
C.69	M.C.	+	"	" "	5.2	4.6	4.3	4.2	0.0	0.1	0.1	0.1
M.B.	L.C.	+	"	" "	4.5	3.8	3.9	4.1	0.0	0.0	0.0	3.4
M.C.	S.C.	+	Clear.	Partially "	2.9	1.2	0.0	1.1	0.0	0.0	0.0	0.0
M.R.	L.C.	+	Turbid.	Firmly "	4.1	3.6	3.8	3.9	0.0	0.0	0.0	3.8

M.26R.F.	L.C.	+	Clear.	Firmly coagulated.		4.4	4.1	4.3	4.0	0.0	0.1	0.0	3.4
M.26R.H.	"	+	"	"	"	4.8	3.6	3.9	4.0	0.0	0.0	0.0	3.0
M.26L.H.	"	+	"	"	"	4.3	3.8	4.0	3.7	0.1	0.0	0.0	3.1
M.29	M.C.	+	Turbid.	"	"	4.6	4.3	3.6	4.3	0.0	0.1	0.0	3.2
M.41	S.C.	+	"	"	"	4.8	4.0	4.2	4.1	0.1	0.1	0.0	0.1
M.43	"	+	"	"	"	4.7	3.3	3.1	4.2	0.0	0.1	0.0	2.9
M.47	L.C.	+	Clear.	"	"	4.1	3.9	3.5	3.1	0.0	0.0	0.1	2.7
M.57	S.C.	+	Turbid.	"	"	3.8	3.6	3.1	3.7	0.1	0.1	0.0	1.0
M.59	M.C.	+	"	"	"	3.7	3.9	4.0	3.8	0.0	0.0	0.1	1.7
M.60	"	+	"	"	"	4.0	3.2	3.0	3.6	0.0	0.1	0.0	0.0
M.74	"	+	"	"	"	4.0	3.2	3.5	4.1	0.1	0.0	0 0	0.0
M.84	S.C.	+	"	"	"	4.6	3.8	3.9	4.3	0.0	0.0	0.0	3.2
M.85	M.C.	+	"	"	"	4.3	4.2	3.6	4.1	0.1	0.0	0.0	0.1
M.89	"	+	"	"	"	4.4	3.6	3.7	3.8	0.1	0.0	0.0	0.0

* Chains of more than twenty elements have been indicated as L.C. M.C. signifies chains of eight to twenty. Shorter chains of six or eight have been recorded as S.C.

The virulence of the hemolytic strains for rabbits was tested by the intravenous injection of 1 cc. of a 24 hour bouillon culture. In two rabbits localizations in the joints occurred after injections of Streptococcus C.63A. and Streptococcus M.B. Strains M.R., C.67A., C.67B., and M.43 produced only slight febrile reactions extending over a few days. Other strains failed to affect the general condition of the animals.

The hemolytic streptococci resemble the non-hemolytic types in regard to their fermentative action. They fall into the two broad groups previously mentioned, those attacking salicin and those unable to do so. Previously it had been shown that the non-hemolytic streptococci isolated from mastitis, regardless of their fermentative activity, would clump when mixed with an agglutinin produced by the immunization of a cow with a single typical strain. This serum did not contain agglutinin for hemolytic bovine strains. It was deemed advisable to ascertain whether this held true with the hemolytic types. A rabbit was immunized with cultures of Streptococcus C.53. Its serum was used to test the agglutinability of the hemolytic streptococci. Blood serum obtained prior to the first injection of the culture failed to contain agglutinin for three typical strains of streptococci.

1 per cent salt solution suspensions of 24 hour horse serum agar cultures were shaken for 20 minutes in a mechanical shaker and diluted with salt solution to a uniform density. To each cubic centimeter of suspension various amounts of antiserum were added. Readings were made after incubation for 24 hours at 38°C. A control tube was always incubated.

Table III indicates the agglutination titer of the streptococci isolated from the udder when tested with the serum of a rabbit immunized with a single strain.

Twenty-six of the twenty-eight strains were agglutinated by the antiserum produced by the immunization of a rabbit with a single typical strain. Streptococci C.65B., C.67C., and M.C. failed to agglutinate at dilutions of 1:100, although the first two possessed practically all characters in common with the type used in immunization. Streptococcus M.C. differed considerably from the others in its low acid production in dextrose, lactose, and maltose and its failure to

TABLE III.

Agglutination Titer of Streptococci Tested with a Serum Produced by the Immunization of a Rabbit with a Single Strain.

Strain No.	Dilutions.				
	1:100	1:200	1:500	1:1,000	1:2,000
C.53	+++*	+++	+++	+++	+
C.57	+++	+++	++	−	−
C.59	+++	+++	+++	+++	++
C.63A.	+++	++	+	−	−
C.63B.	+++	+++	++	+	−
C.65B.	−	−	−	−	−
C.67A.	+++	+++	+++	++	+
C.67B.	+++	+++	+++	+	−
C.67C.	−	−	−	−	−
C.67E.	+++	+++	+++	+	−
C.69	+++	++	+	−	−
M.B.	++	+	−	−	−
M.C.	−	−	−	−	−
M.R.	+++	+++	+++	+++	+++
M.26R.F.	+++	+++	+++	+++	+++
M.26R.H.	+++	+++	+++	+++	+
M.26L.H.	+++	+++	+++	++	−
M.29	+++	+++	++	−	−
M.41	+++	+++	+++	++	+
M.43	+++	+++	+++	+	−
M.47	+++	+++	+++	+++	+
M.57	+++	+++	+++	++	+
M.59	+++	+++	+	−	−
M.60	+++	+++	+++	++	+
M.74	++	+	−	−	−
M.84	+++	++	+	−	−
M.85	+++	+++	+++	+++	++
M.89	+++	+++	+++	+++	+

* +++ indicates a complete agglutination, ++ a strong agglutination without entire clearing of the fluid, + a moderate clumping, and − no agglutination.

ferment saccharose. It is interesting to note that the non-salicin-fermenting strains agglutinated nearly as well as the others. Three cultures of hemolytic streptococci of human origin failed to agglutinate at serum dilutions of 1:100.

DISCUSSION.

While invasion of the udder with hemolytic streptococci has not been observed so frequently as infections with non-hemolytic types, nevertheless serious losses occur from these infections. Of the nineteen cows under observation for an extended period, eight have been disposed of because of the severity of the involvement. In seven others the condition has become chronic. The other four have recovered.

The udder may become invaded at any time during the lactation period. Five cows developed mastitis shortly after calving.

The difficulty of tracing infections was pointed out previously in the investigations of the non-hemolytic types. Doubtless the principal method of entrance is through the teat canal. Injuries play only a minor part as a predisposing factor. In no instance has it been possible to associate injury with this type of disease.

One point stands out clearly, however, and that is that animals suffering from mastitis in one quarter associated with hemolytic streptococci frequently shed identical streptococci in the milk from other quarters. Often the organisms are in pure culture or make up the bulk of the flora of that quarter. These invasions rarely produce gross changes in the gland and frequently fail to change the character of the milk. Cows affected in this way are of considerable danger to other animals in the herd. The milker is usually warned of the infectious nature of the secretions of the diseased quarter, but may be careless in handling milk from the apparently normal quarters. This may explain one ready source of infection.

The development of mastitis in five cows shortly after calving is suggestive of infection with streptococci from the genital tract. Frequently one observes more or less discharge from the vulva of cows following parturition. In four of these cases mastitis developed in the right hind quarter; in the fifth both hind quarters became involved. The genital secretions after calving may readily run over the hind teats, and contained organisms in this manner may gain access to the teat duct.

The pathogenicity of hemolytic streptococci obtained from cases of mastitis to species other than bovines is undetermined. The rela-

tively low virulence of freshly isolated cultures injected into rabbits may be of considerable significance. Raw milk from large dairies must contain a considerable number of these organisms. Usually only the milk from clinically affected quarters is discarded, but the secretion from the other quarters not visibly involved which often contains large numbers of streptococci enters the milk supply. If any considerable number of these organisms were pathogenic for consumers of milk, septic sore throat would occur with considerable frequency; nevertheless, milk containing a few flocculi and hemolytic streptococci obtained from cows which fail to show gross lesions must be looked upon with suspicion until more is known of the source and nature of the virus.

SUMMARY.

Hemolytic streptococci produce more or less severe inflammations of the udders of cows. Frequently infected quarters are swollen, firm, hot, and tender. In a number of instances it has not been possible to detect gross changes in the mammary gland.

The streptococci isolated from the invaded quarters have produced clear zones of hemolysis immediately surrounding the colonies when cultivated in horse blood agar plate cultures. The hemolytic zone has varied from a clear, narrow band up to zones 1.7 to 2 mm. wide.

When the streptococci are classified according to their action upon carbohydrates, they fall into two broad groups: the larger consists of nineteen strains fermenting dextrose, lactose, saccharose, maltose, and salicin; and a smaller number comprising ten species produces acid in dextrose, lactose, saccharose, and maltose and fails to ferment salicin. One of the non-salicin-fermenting strains did not attack saccharose. In no instance was acid production noted in raffinose, inulin, or mannite.

All streptococci except three were agglutinated by an antiserum obtained from a rabbit immunized with a single strain.

Freshly isolated cultures when injected intravenously into rabbits possess but slight pathogenicity. Localizations in the joints occurred in two instances. The others either failed to affect the general condition of the animals or produced only a slight febrile reaction.

[Reprinted from The Journal of Experimental Medicine, September 1, 1918,
Vol. xxviii, No. 3, pp. 333–344.]

A PLEOMORPHIC BACILLUS FROM PNEUMONIC LUNGS OF CALVES SIMULATING ACTINOMYCES.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

Plates 25 to 28.

(Received for publication, June 14, 1918.)

During investigations upon the diseases of young calves, a mild epidemic of pneumonia in calves over 4 weeks old was encountered in the winter of 1917. A few scattering cases occurred during the remainder of the same year. Only one case occurred in the winter of 1918. The character of the lesions in these cases was the same and suggested some one underlying etiological agent.

The amount of lung tissue involved varied considerably from case to case. In all at least one of the smaller lobes (cephalic, ventral, azygos) was affected. Usually all were pneumonic. More rarely, in addition, the adjacent portions of the large caudal lobes were involved. The distribution was with few exceptions symmetrical. The trachea and bronchi usually contained soft, opaque, whitish masses embedded in mucus. When one of the affected lobes was cut across, a pearly white, thick, mucoid mass slowly oozed out of the cut ends of the small bronchioles within the diseased region. The affected lobes were slightly or considerably larger than in the normal collapsed state. The tissue was bright reddish and permeated with grayish 1 to 2 mm. foci, closely set. The texture of these did not differ appreciably from the rest of the tissue. Sections of the diseased lobes indicated a suppurative bronchopneumonia, with some fibrin in the most recently invaded tissue.

This brief description applies to the recent acute stage, such as was found in animals killed when dyspnea was marked. Older stages of the disease with a considerable change of the picture given above were found in several calves, 3 or more months of age. In these, circum-

scribed portions of lung tissue had become encapsuled abscesses. Leaving a more thorough description of this and other types of pneumonia for a later communication, we will consider here only one kind of microorganism encountered whose claim to be the primary or exclusive etiological factor need not be insisted on until more cases have been studied with the culture methods thus far successful in bringing it to the surface.

The organism to be described possesses for the bacteriologist an interest quite apart from its etiological relation to the disease process associated with it. Owing to the peculiar morphological and cultural characters, a description is best begun with the most striking stage which is its growth at about the 3rd day in tubes containing coagulated blood serum and sealed with sealing wax.

The method of sealing tubes is an outgrowth of the Nowack method of cultivating *B. abortus* in a sealed jar with cultures of *B. subtilis*. In work done in 1911[1] it was found that the Nowack procedure could be simplified by directly connecting the tube containing *B. subtilis* with the culture of *B. abortus* by means of a rubber tube or by simply sealing the culture tube. This latter method has proved uniformly satisfactory in cultivating *B. abortus*.

If bits of the involved lung tissue about 3 to 4 mm. in diameter are placed in the condensation water of coagulated horse serum and the tube is sealed with sealing wax, there will be noticed after several days minute, whitish flocculi in the condensation water best seen by transmitted light when the tube is tilted or shaken. The flocculi vary from mere specks to 1 mm. in diameter according to age and the special characters of the culture medium (Fig. 6).

Under a low power one of these bodies appears as shown in Fig. 1. An irregularly spherical mass is made up on the periphery of club-shaped, expanded, roundish, or pear-shaped bodies. Similar bodies appear when the focus is raised. Evidently the entire flocculus is made up of radiating filaments ending in clubs. The filaments are usually obscured by the clubs. Besides the latter, the flake contains

[1] Smith, T., and Fabyan, M., Ueber die pathogene Wirkung des *Bacillus abortus* Bang, *Centr. Bakteriol., 1te Abt., Orig.*, 1912, lxi, 549. Fabyan, M., A contribution to the pathogenesis of *B. abortus*, Bang.—II, *J. Med. Research*, 1912, xxvi, 441.

minute, irregular masses of crystals, shown as black objects in Fig. 1. The crystals are very minute and probably bunches of acicular bodies. Under a high power the clubs appear as slightly refringent bodies with a sharply defined limiting membrane (Fig. 2). Not infrequently in small flocculi cylindrical filaments not enlarged at the ends are seen growing out among the clubs. The latter are about 10 microns in diameter. Within certain terminal bodies a second and even a third membrane is visible. The terminal filaments and clubs are flexible and are gently moved to and fro in currents under the cover-slip The structure of the more central parts of the flocculus cannot be made out owing to the density of the peripheral zone. When crushed the body becomes a mass of detritus in which occasionally filaments, about 2 microns broad, are seen as parallel strands very faintly outlined.

The impression gained at first from a study of the unstained flocculi in the fluid in which they grow is that the culture consists of a filamentous organism whose frequent branching produces the roundish flake. If flocculi 2 to 4 days old are allowed to dry undisturbed upon cover-slips, then heated and stained in the usual way with some aniline dye, such as alkaline methylene blue, for several hours and decolorized in 0.01 per cent of acetic acid to remove the dye from the background of the specimen, it will be seen that the spot covered by a flake is unstained. Its interior is, however, occupied by a tangle of very fine, segmented filaments made up of bacilli. There is no evidence of branching (Fig. 4). The bacilli measure from 0.4 to 0.5 microns in diameter. They stain much as the common bacteria do in alkaline methylene blue and diluted carbolfuchsin, but are decolorized according to Gram. As stated, the flocculi themselves fail to take any stain and appear white when the background of the film is still tinted with the stain.

The above type of culture on horse serum has been maintained in one case for many generations. The growth was materially improved when about 1 cc. of calf serum water was added to the condensation water present. All the tubes have been sealed. Multiplication was not evident in open tubes. In subcultures, after several weeks either at 37°C. or at room temperature, the slanted surface may or may not become covered with very minute, elevated, pointed-like colonies

which, on microscopic examination, have the same characters as the flocculi in the condensation water. All serial cultures have been obtained by transferring several loops or a drop of the condensation water to a fresh tube and not from the surface colonies. When the culture medium is not very favorable, the flocculi remain very small and the clubs become large and even spherical in outline, or else the flakes become quite large.

After 3 or 4 days changes begin to set it. One of the most important is shown by staining. The flocculi now begin to take up some of the stain and the material of which they are made up has a striated or lamellated appearance, as if some substance had been deposited within the membrane. Peculiar, bizarre forms are the result. The nearest objects to which they may be compared are the myelinic or so called Buhl's bodies seen in fresh unstained sputum originating from the alveolar epithelium of the lungs. At this stage the stained film no longer gives the clear picture of the contents of the flake. Bacteria are no longer detected in most of the flakes. They have either been dissolved or are no longer accessible to the stain or else are obscured by the absorbed dye in some of the clubs. To see the chains of bacilli within the flakes it is therefore necessary to stain early vigorous stages of growth.

After some months of cultivation, with transfer intervals of about 5 days, in horse serum tubes, or rather in the condensation water of such tubes, certain changes have been observed in some substrains which permanently affect the substance enveloping the bacilli and which are presumably degenerative in character.

The clubs of the flocculi become surrounded with a layer of fine granules and eventually concealed by them. The granules are slightly refringent up to 1 micron in diameter and very variable in size. They become detached from the flakes and form small groups which collect at the surface of the condensation water as a white, creamy layer. The appearance and physical characters suggest some fatty substance. Osmic vapor stains them feebly brownish. Scharlach R placed on a dried film is absorbed by the material which becomes well tinted. Similarly, a dried film stained with the fat dye in the usual way for sections and mounted in water shows imbibition of the dye. However, similar effects are obtainable with alkaline methylene blue and the specificity of the fat stain remains questionable.

Cultures upon slanted agar were obtained in two ways, (*a*) from lung tissue direct and (*b*) from the horse serum tubes.

(*a*) Growth upon slant agar tubes to which particles of diseased lung tissue had been added and the tube sealed with sealing wax occurred quite regularly. In some cases a film covered the agar surface just above the tissue in the condensation water. On others besides this growth, scattering 1 to 2 mm. yellowish colonies appeared on the slope. The colonies are roundish, slightly raised masses.

When examined under a high power fresh the colony was found to be composed of roundish, ring-like, rather faint bodies about 2 microns diameter (Fig. 5). On the periphery or near the center of the body is a very minute refringent speck. The appearance suggests a spore within some material of low refringence. Staining dried films did not give more information. The disc-like bodies became more strongly outlined by a feeble peripheral stain but no staining equivalent to that of ordinary bacteria was obtained.

Subcultures of such colonies under the same conditions failed to multiply. Tentatively the explanation was that the organisms sporulated and failed to germinate in a second tube. Transfers to other media also failed.

(*b*) After the serum cultures had become established so that multiplication occurred regularly in subcultures, transfers to slanted agar were frequently made. These were only occasionally successful and from such transfers series of agar cultures were maintained. The medium contained a bit of guinea pig spleen and some calf serum water added to the condensation water on hand. These tubes also were kept sealed. The colonies were very minute, drop-like, discrete, or else fairly large, *i.e.* 1 to 1.5 mm. diameter, or else both minute and large colonies appeared on the same slanted surface (Fig. 7). A smooth continuous layer of growth usually started from the condensation water and extended 5 to 10 mm. upwards on the slant. The substance of the large colonies was more or less coherent since the colony could be moved along or picked up with a wire *in toto*. In films made from such colonies, which were in this as well as in former series of a straw color, the growth was found always to be bacilli with no indications of a capsular substance. The bacilli were lying close together. There was slight variation among the bacilli, both as to width and length and staining capacity, from the same colony (Fig. 8).

After 1 or 2 weeks growth on agar slants, the condensation water either remained clear, or else a thin, creamy layer formed at the surface. No flocculi appeared in it at any time. In the meantime changes went on in the growth, leading to the disappearance of the bacillus type and the appearance of the coccus type as found in original agar cultures from diseased lung tissue (Fig. 5). This occurred after 7 days and the process was completed in about 10 days (Figs. 9 and 10).

The coccus type, therefore, must represent a definite stage in the growth cycle of this microorganism. Does it represent a minute organism enclosed in a feebly staining capsule, and if so, is this minute body an endospore or an arthrospore? No definite answers can be given to these queries at present from the morphological standpoint, owing to the minuteness of the bodies. In one case in a fresh, unstained film, what appeared to be endospores were found both at the ends and in the course of the filaments. These bodies were markedly refringent and very slightly bulged the walls of the rods outward. Further culture studies may throw some light on the nature and function of these bodies.

In addition to the horse serum and agar media described, a variety of culture media was tried without success. Either the multiplication was feeble or else absent altogether. Among the media tried were ordinary slanted agar, milk and potato tubes, sealed and unsealed; bouillon plus bits of tissue, covered with paraffin oil; ascitic fluid plus tissue and paraffin oil; and fermentation tubes of bouillon plus tissue. Calf serum water by itself produced only a very feeble growth.

Evidently the multiplication depends upon certain definite substances in culture media in a given, slightly reduced, oxygen tension. Prolonged cultivation may in time bring the organism to multiply in the ordinary culture media, but this stage has not been reached after 5 months of continuous cultivation on special media.

Naturally, in meeting the peculiar morphological entities in cultures, one would at first regard them as contaminations, and this assumption, as stated above, retarded the work of isolating and identifying the microorganism for some time. When bits of animal tissues are used both for purposes of inoculation and for stimulating growth the

question of mixed cultures must be kept constantly in sight. In the work before us, the culture tubes containing agar plus guinea pig spleen had been in the incubator from 2 to 4 weeks, sealed, and then in room temperature for another period of 1 to 2 months before they were used. A chance for any contaminated tube to slip through unnoticed may be regarded as almost negligible. When bits of diseased lung tissue were inoculated—and this is probably the only way for securing growth of the organism—the results were very uniform. When the organism was present alone, all cultures contained the same growth. When other bacteria were associated they were usually present in all cultures and suppressed the growth of the former. This was then recognizable for a short time in the condensation water by its peculiar growth forms.

Inoculations of relatively large quantities of culture material into the peritoneal cavity of mice, guinea pigs, and rabbits have not produced anything beyond slight local changes. In several guinea pigs and one rabbit one, two, and three doses were given, separated by intervals of weeks in order to develop any susceptibility to subsequent inoculations. No multiplication was evident beyond slight local peritoneal opacities and thickenings, except in one rabbit which received three separate intraperitoneal injections and was chloroformed 16 days after the last injection. A small, encapsulated abscess about the size of a pea was found attached to a coil of the colon. This was removed and crushed and portions of the contents were added to tubes of solidified horse serum. From this the *Actinomyces*-like organism was recovered and successfully subcultured.

This organism has been identified microscopically in cultures from ten cases of pneumonia having common anatomical and histological characters. Owing to the confusing pleomorphism in different culture media and at different ages of the same culture, it was not identified in the three earliest cases and the cultures regarded as mixtures. The notes describing the forms seen were, however, sufficient to make a diagnosis possible later on. In the subsequent cases the peculiarities of the serum cultures aroused attention but they were not at once identified with the original agar cultures from the same tissues. The organisms in the serum tubes from three cases were kept alive through several generations but owing to interruptions in the work and the

apparently feeble vitality of the organism they were lost. Finally cultures from one of the last cases have been maintained through many generations. Unfortunately the supply of material which was abundant in the winter of 1917 became scarce soon after, so that the successful methods for continuous cultivation over long periods could be used on only one case.

Relationship of the Pleomorphic Bacillus to Bacteria Already Described.

The suggestion which occurs to one seeing this bacillus for the first time is that it is related to or identical with one or another of the *Actinomyces* types cultivated by Bostroem, Wolff and Israel, J. H. Wright, and others. This suggestion is soon dispelled. The organism is a minute bacillus, growing in chains, the chains held together by intercellular substance. There is no branching and the Gram stain is negative. The peculiar *Actinomyces* type of growth obtained suggests the possibility, however, that when the proper culture medium is found the ray fungus itself will be cultivated with clubs as it occurs in animal tissues. The rare and uncertain appearance of clubs in *Actinomyces* cultures has led observers to infer that the clubs may at least in part be formed by the host tissues. The observations upon this bacillus suggest the compromise theory that the clubs are formed by the bacillus and later impregnated with tissue fluids. The absence of any capacity for holding dyes on the part of the clubs in the cultures of the bacillus and the relative ease with which acid dyes are held by the clubs of the *Actinomyces* grains as obtained from pus tends to support this view.

In looking over the literature to find some types which might be assimilated to the described bacillus, the writer found a publication by Lignières and Spitz[2] which seems to supply the missing link. These authors describe an affection which prevailed as an epizootic in Argentina in 1900 and 1901. It was at first regarded as true actinomycosis but the failure to stain the filaments in pus with accepted methods and negative cultures on potato induced Lignières to undertake an investigation.

The disease assumes various forms. (1) As an abscess of the subcutaneous tissue of the throat. The abscess develops very slowly and is associated with

---

[2] Lignières, J., and Spitz, J., Actinobacillose, *Rev. Soc. mêd. arg.*, 1902, x,, 105 9 plates.

little pain. It eventually ruptures and discharges a very thick, sticky, pasty pus. Abscesses may also appear over the parotid gland, the superior maxillary bone, at the base of the ear. The disease has been found to occur in many other parts of the body with involvement of the regional lymph nodes. (2) As an affection of the tongue which is clinically identical with the affection known as wooden tongue (*langue de bois*), as an affection of the pharynx, the parotid gland, the mammary gland, and as localizations in the lungs leading to lesions resembling tuberculosis. Hepatization may also occur. Lesions of the bones were rarely found. They were located in the superior and inferior maxillary bones and gave rise to appearances formerly described as osteosarcoma. That form described first as cold abscess of the neck makes fully 80 per cent of all cases; localization in the tongue 5 per cent.

Lignières found in the pus, either directly or after treatment with caustic potash and centrifuging, radiating masses of clubs which did not show any mycelium and failed to stain according to Gram. Acid dyes, like picric acid, gave a distinct coloration to the clubs.

When pus from closed abscesses is ground up and tubes of agar-agar inoculated with it, growth is evident at 37°C. within 24 hours. The organism may appear in early cultures as a bacillus, later like a diplococcus or a streptobacillus, and in old cultures as bizarre involution forms. It multiplies in plain, glycerol and sugar bouillon, giving the latter a plainly acid reaction. In gelatin the growth is very feeble. Liquefaction does not occur. On agar the colonies, if crowded, are small, translucent, bluish; larger, opaque when scarce. Activity of growth increases with successive transfers and may become like and nearly as abundant as that of the typhoid bacillus. Milk is regarded as a good medium but coagulation does not occur.

Lignières' strain was of a relatively high virulence. A culture on agar injected into the peritoneal cavity of guinea pigs killed in 12 to 24 hours, the lesions being those of an acute peritonitis. Rabbits were far less susceptible. In the mouse a subcutaneous inoculation produced only a transient induration. In cattle a local abscess was always produced after subcutaneous inoculation.

The organism of Lignières and Spitz presents many features that are like those of the bacillus described. The fundamental one is that an *Actinomyces*-like organism gives rise to a bacillus in cultures whose pleomorphism agrees well with that found by the writer on agar slants. The differences between the two organisms are, however, numerous. Lignières' bacillus grew well on ordinary media and in open tubes. The one described here could not have been cultivated in this way. Lignières found the *Actinomyces*-like characters in pus from tissues but failed to reproduce them in cultures. The reverse is the experience of the writer who found the organism in the lungs in the form of very fine bacilli. Lignières traced his organism in a

variety of lesions in adult cows, whereas the bacillus here described was associated with a very characteristic and extensive broncho-pneumonia of young calves.

On the whole, the writer is inclined to regard Lignières' bacillus as identical with his own with such variations as are likely to appear within the boundaries of any species or strain, widely separated geographically and attacking animals of different age periods.

The name given by Lignières and Spitz to their bacillus—actino-bacillus—involves the establishment of a new genus. This is hardly justifiable when we consider that the characters are given to the group by a variable and disappearing physiological factor analogous to a capsule or capsular material. The writer suggests a new species name—*Bacillus actinoides*—for the culture here described and leaves it to future investigations to determine whether it is specifically identical with Lignières' actino-bacillus or not.

Following the publication of Lignières and Spitz, Nocard[3] was able to confirm their results on cases of wooden tongue of cattle in France. Higgins[4] described four cases of "actino-bacillosis" in cattle (tumor of the region of the pharynx, abscess of parotid, disease of the tongue, and a growth on the jaw). Neither of these authors found any difficulty in isolating and cultivating the bacillus. Neither obtained any *Actinomyces* forms in their cultures. According to Higgins, guinea pigs inoculated into the peritoneum die of a generalized infection in 19 to 31 days. Rabbits are also susceptible and a generalized eruption follows intraperitoneal inoculation.

Bearing upon the peculiar culture forms of this bacillus a somewhat analogous instance of the dependence on special media may be referred to here. *Leuconostoc mesenteriodes*, a micrococcus causing injurious fermentations in sugar refineries, was studied by Liesenberg and Zopf[5]

[3] Nocard, E., Les maladies microbiennes des animaux, Paris, 3rd edition, 1903, ii, 374, footnote.

[4] Higgins, Actino-bacillosis, *Bull. No. 1, Dept. Agric., Health of Animals Branch, Dominion of Canada*, 1904.

[5] Liesenberg, C., and Zopf, W., Ueber den sogenannten Froschlaichpilz (Leuconostoc) der europäischen Rübensucker- und der javanischen Rohrzuckerfabriken, Beitr. Physiol. u. Morphol. niederer Organismen, 1892, pt. i; abstracted in *Centr. Bakteriol.*, 1892, xii, 659.

who found that while enormous, gelatinous capsules are developed in media containing sugars, these fail to appear in media not containing dextrose or saccharose. In these it appears like an ordinary streptococcus. This measures 0.9 to 1.2 microns in diameter, whereas the capsules may attain a diameter of 6, 10, or even 20 microns.

In the case of the bacillus here described the formation of the flocculi with terminal clubs is evidently dependent on some substance in blood serum which survives the coagulating temperature of 70–75°C.

SUMMARY AND CONCLUSIONS.

A bacillus was found associated in pure culture with an extensive lobar bronchopneumonia in calves. It occurs in the exudate as a minute bacillus in small groups. In cultures it appears in three forms: as a bacillus, as a coccus-like endospore or arthrospore, and as a conglomerate *Actinomyces*-like flake or colony with peripheral clubs. The bacillar and coccoid forms occur on agar, the *Actinomyces* form in the condensation water of coagulated serum (horse). The coccoid form is probably a spore state, the minute refringent spore being contained in a roundish, unstainable mass representing either the remnants of bacillar substance or some capsular material. The somewhat striking similarities between this organism and *Actinomyces* are expressed by the massed growth with terminal clubs, the bacillar and coccoid stages, all of which are characteristic of *Actinomyces*.

Sealing the tubes is essential for multiplication. Cultures must be renewed within a few days, otherwise multiplication fails. The substance which forms the bulk of the radiate flocculi is probably of capsular nature, greatly overproduced in serum tubes and scarce or absent on agar. Its nature is unknown.

The organism is not appreciably pathogenic when injected into certain small laboratory animals.

EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. A flake or colony with peripheral clubs from the condensation water of a horse serum culture. The blackish specks on the colony are masses of acicular crystals. The flake is flattened out between slide and cover-glass. × 120.

FIG. 2. The margin of a flake in condensation water flattened gently between slide and cover-glass. × 1,000.

PLATE 26.

FIG. 3. A flake or colony dried on a slide and stained in alkaline methylene blue. The flake is feebly outlined. The stained masses are bacilli. Thirteenth transfer on horse serum, 2 days old. × 115.

FIG. 4. Margin of a flake from the same culture as the one from which the flake in Fig. 3 was taken. The bacilli appear in chains. The dark bodies are masses of bacilli. × 1,000.

FIG. 5. Colony from an original agar culture from the lungs as described in the text. × 1,000. The film is unstained and spread between slide and cover-glass in fluid from the culture. Coccoid bodies containing each a highly refringent granule.

PLATE 27.

FIG. 6. Sealed culture on horse serum showing the flocculi or colonies in suspension in the condensation water. Some have become attached to the sides of the tube as a result of shaking the tube.

FIG. 7. Sealed culture on an agar slant containing in the condensation water a piece of guinea pig spleen and some calf serum water. Culture 7 days old. After 5 months of artificial cultivation (about thirty transfers).

FIG. 8. Film stained in alkaline methylene blue from an agar slant plus guinea pig spleen about 48 hours old. Note only rod forms. Some of these are more intensely stained and a trifle thicker than the great mass. × 1,000.

PLATE 28.

FIG. 9. Film from a culture on the same kind of medium as given for Figs. 7 and 8. 6 days old. Alkaline methylene blue. Among rod forms are roundish coccus-like, feebly stained bodies. × 1,000.

FIG. 10. Film from the same culture stained several hours in diluted carbol-fuchsin. The roundish forms of Fig. 9 now appear as deeply stained, smaller bodies suggesting endospores. × 1,000.

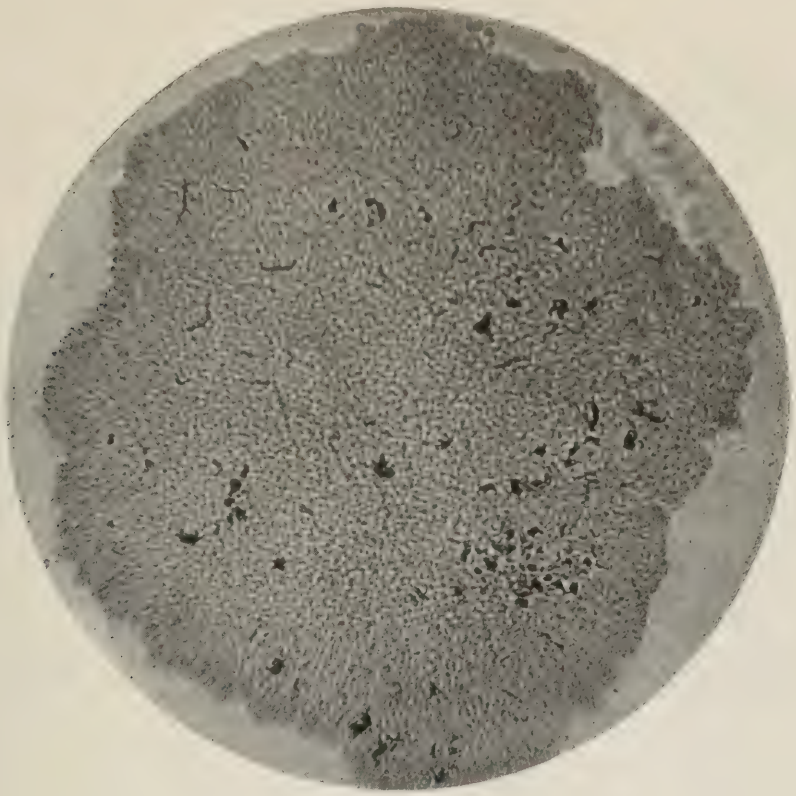


FIG. 1.

FIG. 2.

(Smith: Pleomorphic bacillus from pneumonic lungs.)

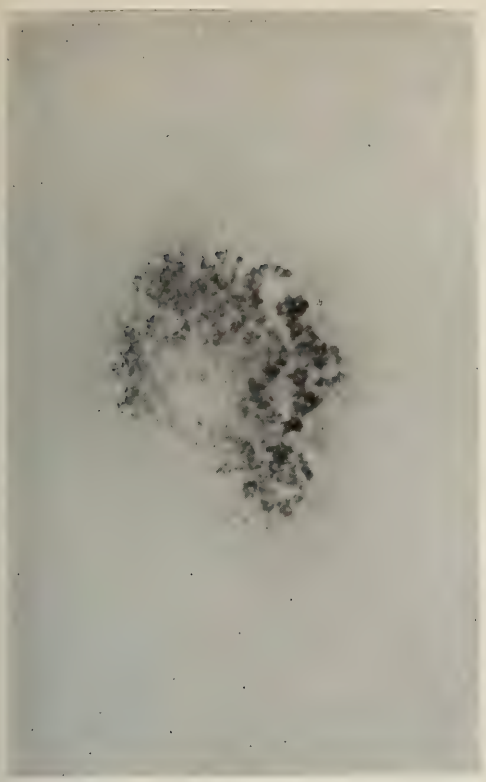


FIG. 3.

FIG. 4.

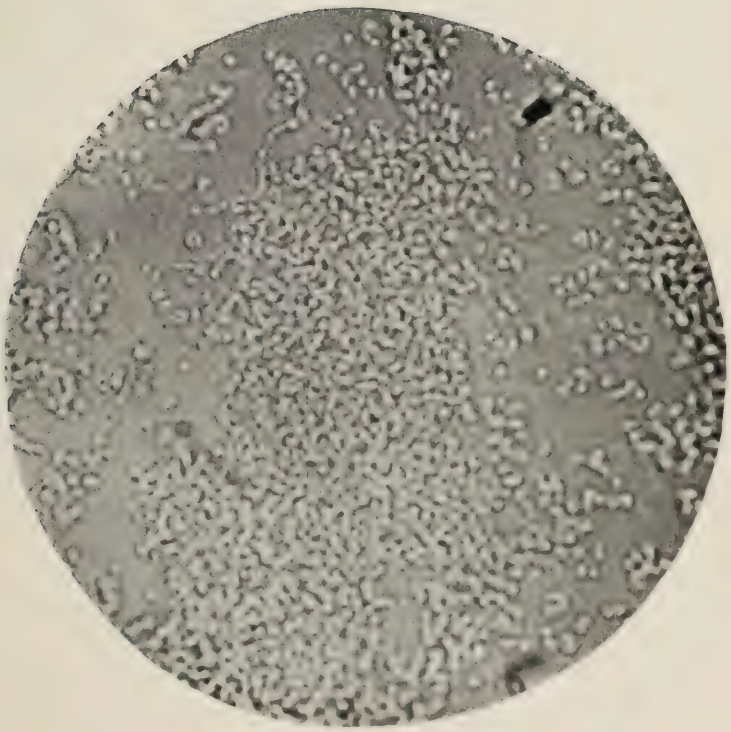


FIG. 5.

(Smith: Pleomorphic bacillus from pneumonic lungs.)

FIG. 6.

FIG. 7.

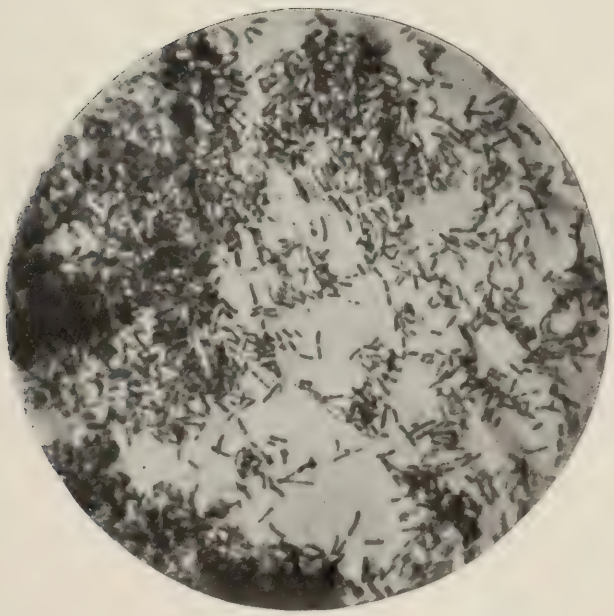


FIG. 8.

(Smith: Pleomorphic bacillus from pneumonic lungs.)

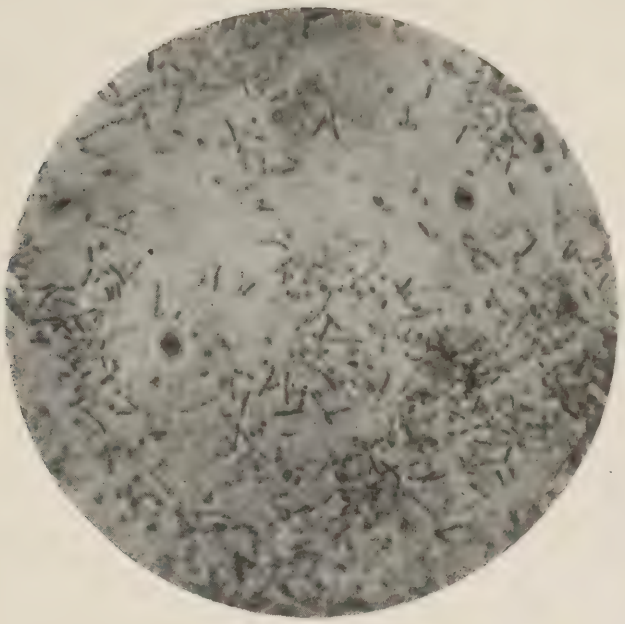


FIG. 9.

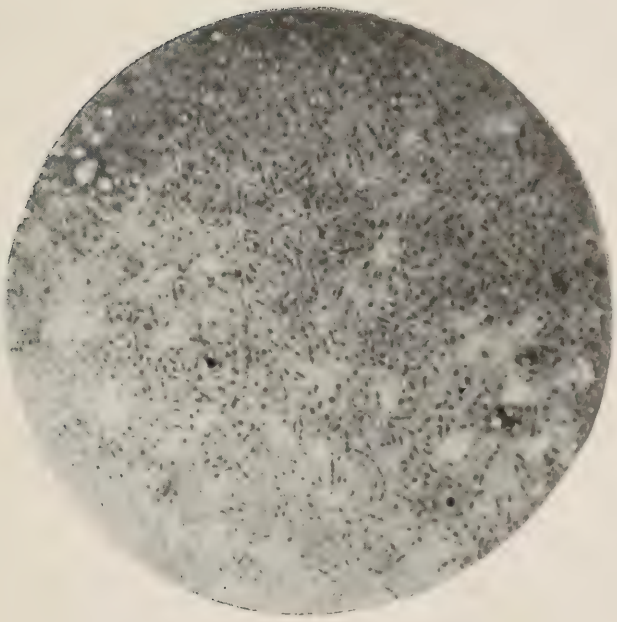


FIG. 10.

(Smith: Pleomorphic bacillus from pneumonic lungs.)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, November 1, 1918,
Vol. xxviii, No. 5, pp. 585–605.]

AN IMPROVEMENT IN THE METHOD OF ISOLATING AND RECOVERING THE BACILLUS OF CATTLE ABORTION THROUGH GUINEA PIGS.

BY ERNEST W. SMILLIE, D.V.M.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, July 15, 1918.)

History of the Test.

For the isolation of *B. abortus* Bang up to 1912 there were available the culture methods of Bang and Nowak. Early in 1912 Theobald Smith and Fabyan[1] showed that *B. abortus* inoculated into guinea pigs produces a disease with well defined characters, and that the disease can be isolated from these guinea pigs after 3 or more months. This method proved a valuable addition inasmuch as culture methods frequently failed when badly soiled fetal membranes were objects of investigation.

This inoculation disease had already been recognized as an entity, distinct from tuberculosis, in 1894 by Smith,[2] and Schroeder and Cotton[3] had the disease under observation since 1911 ascribing it, however, to a Gram-positive bacterium. This error was rectified in a publication issued in March, 1912.[4] The disease in guinea pigs was more fully described by Fabyan[5] in 1912. Since that date the guinea pig has been used by various workers for the isolation of *B. abortus* from fetal membranes and fetal tissues.

In view of the value of the method it seemed desirable to determine whether it might be possible to shorten the life period of the inoculated guinea pig without impairing the chances for obtaining cultures. In the following pages a number of experiments are described which were planned with this object in view.

[1] Smith, T., and Fabyan, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1912, lxi, 549.

[2] Schroeder, E. C., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 7*, 1894, note by T. Smith, p. 80.

[3] Schroeder, E. C., and Cotton, W. E., *Proc. Am. Vet. Med. Assn., 48th Convention*, 1911, 442.

[4] *U. S. Dept. Agric., Bureau Animal Industry, Circular 198*, 1912.

[5] Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.

EXPERIMENTAL.

The material used to inoculate guinea pigs was derived from pure cultures as well as from tissues of fetuses and fetal membranes.

The guinea pigs were inoculated either into the subcutis or the abdominal cavity. When the animals were considered ready for bacteriological examination they were chloroformed, autopsied, the lesions noted, and the following procedure, which is the one regularly used in this laboratory for the cultivation of *Bacillus abortus*, was adopted throughout.

Ordinary veal peptone agar tubed and slanted is the medium employed. The surface of the organs from which cultures are to be made is, if necessary, seared with a heated spatula. Bits of tissue about the size of split peas are then torn out of the organ with sterile forceps, rubbed over the entire surface of the agar with a platinum loop, and finally pushed down into the condensation water. The cotton plugs are clipped off level with the tops of the tubes. The tubes at this point are thoroughly heated in the flame to kill any spores adhering to the contained cotton plugs as a result of handling, and after the tubes have cooled somewhat they are hermetically sealed with a layer of sealing wax. They are then incubated at 37°C. Colonies of *Bacillus abortus* are usually observed on the agar slant after 5 to 10 days incubation.

Cultures were prepared from lungs, liver, kidneys, and spleen in all cases; from the superficial inguinal lymph nodes when the animal had received a subcutaneous injection; from the retrogastric lymph nodes when the injection was intraabdominal; and from the testicle and the ovary or uterus. After an incubation of from 6 to 10 days, the cultures were examined and any growth was noted and studied. The colonies were counted on the agar surface. In case no growth appeared, the condensation water containing the bit of tissue was shaken so as to cover the agar surface and the tubes were replaced in the incubator. The object of this procedure was to give any bacteria growing out of the bit of tissue or in the condensation water an opportunity to multiply on the inclined surface. Probably one out of every ten to twenty tubes responded to this treatment.

To identify *Bacillus abortus* the colonies were examined both macroscopically and microscopically. The macroscopic characteristics of

such colonies are in themselves almost diagnostic. They vary considerably in size according to the numbers on the agar surface. When crowded they are relatively minute and as a rule discrete, except near the margin of the condensation water. When 1 to 2 cm. apart, they may become 3 mm. in diameter and strikingly convex. The microscopic examinations of these colonies were made in all instances to verify the macroscopic inspection, and the Gram test was applied to differentiate *Bacillus abortus* from Gram-positive organisms, such as *Bacillus pyogenes*.

The diagnosis of infection with *Bacillus abortus* was furthermore strengthened by a macroscopic examination of the organs of every guinea pig. As is now well known, in positive cases the spleen is much enlarged, highly congested, more or less nodular on the surface, and the Malpighian bodies may be enlarged and show through the distended capsule and on section as grayish foci of varying sizes. In addition to this characteristic lesion, one or both testicles may be attacked and the epididymis converted into an indurated, centrally necrotic mass. Minute nodules in the liver are found in most cases, usually not quite a millimeter in diameter, sometimes yellowish and probably representing necroses, sometimes grayish or pearly and then representing minute collections of cells as described by Smith and Fabyan and Fabyan.

Series I.

The first series of guinea pigs injected consisted of twelve, six males and six females, varying between 300 and 400 gm. in weight. They were injected September 26, 1917, with a stock culture recovered from a fetus after passage through a guinea pig. Three of each sex were inoculated subcutaneously and the same number intraperitoneally, each individual receiving 1 cc. of a suspension of the strain. The suspension was prepared by washing off the surface colonies of a 72 hour agar slant with sterile salt solution, diluting the resulting suspension to the approximate density of a 24 hour bouillon culture of *Bacillus typhosus* and then again diluting fifty times. Table I summarizes the results. The enlargement of the spleen is designated in bulk and not in dimensions.

TABLE I.

Results of Inoculation of Guinea Pigs with a Pure Culture of B. abortus Isolated through Guinea Pigs from a Fetus.

Guinae pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
1	Male.	Intraperitoneal.	15	+ 6	Six times normal size; adherent to ribs.	—	Spleen: luxuriant growth. Liver: good " Testicle: " "	+
2	Female.	Subcutaneous.	15	+14	Five times normal size; hyperemic and friable.	—	Spleen: " " Regional lymph node: good growth. Kidney: good growth. Liver: few colonies.	+
3	Male.	Intraperitoneal.	21	+22	Eight times normal size; easily torn; surface nodular; nodules numerous.	Small necrotic foci in liver.	Spleen: 200 Regional lymph node: 150 Testicle: 18 Lung (contaminated): 18 Kidney: 2	+
4	Female.	Subcutaneous.	21	+13	Eight times normal size, surface nodular.	Small abscess at point of injection.	Spleen: 100 Regional lymph node: 200 Lung: 31 Kidney: 6 Ovary: 1	+

5	Male.	Intraperitoneal.	29	+20	Slightly enlarged; very dark; surface nodular.	Minute necrotic foci in liver.	Spleen: 150 Retrogastric lymph node: 50 Kidney: 7 Lung: 3 Liver: 2	+
6	Female.	Subcutaneous.	29	+52	Six times normal size; surface nodular.	Numerous minute necrotic foci in liver.	Spleen: 150 Regional lymph node: 125 Lung: 4 Ovary: 3 Liver: 2	+
7	"	Intraperitoneal.	35	−15	Eight times normal size; adherent to peritoneum and left kidney; tubercle in spleen at adherent point.	Few minute necrotic foci in liver. Capsule of left kidney thickened.	Spleen: 300 Retrogastric lymph node: 300 Kidney: 300 Lung: 6 Liver: 3 Ovary: 2	+
8	Male.	Subcutaneous.	35	+87	Markedly enlarged; easily torn; rounded borders.	Liver enlarged; minute scattering tubercles.	Spleen: 14 Regional lymph node: 140 Lung: 9 Testicle: 6 Kidney: 5 Liver: 2	+
9	Female.	Intraperitoneal.	43	−29	Nine times normal size; surface smooth and easily torn.	—	Spleen: 100 Retrogastric lymph node: 5 Kidney: 8 Lung: 5 Liver: 2 Uterus: 2	+

TABLE I—*Concluded.*

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
10	Male.	Subcutaneous.	42	+90	Twice normal size; hyperemic and surface nodular.	One tubercle in liver 3 mm. in diameter.	Spleen: 80 Regional lymph node: 60 Liver: 3 Kidney: 3 Testicle: 2 Lung: 0	+
11	Female.	Intraperitoneal.	52	−35	Enormously enlarged; adherent to peritoneum and left kidney; nodular surface.	Minute scattering tubercles and small necrotic foci in liver.	Spleen: 75 Retrogastric lymph node: 50 Kidney: 12 Lung: 7 Uterus: 4 Liver: 0	+
12	Male.	Subcutaneous.	52	+106	Enormously enlarged; nodular surface; small glistening tubercles.	Minute irregular necrotic areas in liver.	Spleen: 75 Regional lymph nodes: 150 Kidney: 20 Liver: 3 Lung: 3 Testicle: 0	+

Series II.

Six female guinea pigs, weighing between 325 and 375 gm. each, were used in the second series. They were inoculated September 26, 1917, three subcutaneously and three intraabdominally with a culture of *Bacillus abortus*, recovered from the placenta of a cow, after passage through a guinea pig. The method of preparation of the material to be injected and the dosage were the same as for the guinea pigs of Series I. A condensed description of Series II is given in Table II.

Series I and II were inoculated with the strains from a certain fetus and a certain placenta from two herds respectively, to determine whether or not there was an appreciable difference in the pathogenicity. It will be noted from the tables that the extent of the lesions produced, and the comparative colony counts for the two strains are approximately the same. As far as the guinea pig test is concerned the strains are evidently of the same level of virulence.

Series III.

The third series of guinea pigs consisted of six males and six females weighing between 350 and 450 gm. each. Three guinea pigs of each sex were inoculated subcutaneously, and the same number of each sex intraabdominally, on October 24, 1917, with a salt solution suspension of crushed cotyledons from the placenta of another cow.

The material to be injected was prepared in the following manner: Five cotyledons that were apparently affected were removed into sterile Petri dishes. After they had been thoroughly washed in running tap water from a deep well to remove particles of straw, etc., pieces varying in size from 1 to 2 gm. were clipped from each cotyledon, care being taken to cut down to the base of the villi. The bits of tissue were then ground up with sterile quartz sand in a sterile mortar. The ground mass was suspended in 0.85 per cent salt solution until a density was reached which compared with a 24 hour bouillon culture of *Bacillus typhosus*. Each animal was injected with 1 cc. of this suspension. Table III is a summary of the results.

An analysis of the three foregoing experiments indicates that the inoculation disease in guinea pigs due to *Bacillus abortus* runs a fairly definite course. The number of colonies appearing in cultures is

TABLE II.

Results of Inoculation of Guinea Pigs with a Pure Culture of B. abortus Isolated through Guinea Pigs from the Placenta of a Cow.

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
13	Female.	Intraabdominal.	16	−26	Twice normal size; congested; easily torn.	—	Spleen: luxuriant growth. Retrogastric lymph node: luxuriant growth. Kidney: few colonies. Liver: " " Lung: " "	+
14	"	Subcutaneous.	16	+16	Normal in size and appearance.	Minute grayish necrotic foci in liver. Small abscess (8 mm. diameter) at site of injection.	Spleen: 200 Liver: 20 Kidney: 15 Lung: 3	+
15	"	Intraperitoneal.	28	+21	Five times normal size; surface nodular.	Scattering grayish (necrotic) foci in liver.	Spleen: 200 Retrogastric lymph node: 48 Liver: 10 Kidney: thin film. Lung: " "	+

16	Female.	Subcutaneous.	28	+58	Four times normal size; nodular surface.	Minute necrotic areas in liver. Small subcutaneous abscess at site of injection.	Spleen: Regional lymph node: Lung: Liver: Kidney: Ovary: thin film.	175 300 8 4 3	+
17	"	Intraperitoneal.	50	+41	Eight times normal size; congested; surface smooth and easily torn.	Minute (necrotic) areas in liver; walls of uterus injected.	Spleen: Retrogastric lymph node: Kidney: Liver: Lung: Uterus: few.	100 40 4 2 1	+
18	"	Subcutaneous.	50	+116	Four times normal size; marked congestion; small tubercles; nodular surface.	A few scattering nodules in liver.	Spleen: Regional lymph node: Kidney: Liver:	125 10 6 4	+

TABLE III.

Results of Inoculation of Guinea Pigs with Salt Solution Suspensions of Placenta from a Case of Abortion.

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
19	Female.	Subcutaneous.	15	+15	Twice normal size; congested; easily torn.	—	Spleen: 60 Regional lymph node: 200 Kidney: 20 Lung: 1 Liver: 1	+
20	Male.	Intraperitoneal.	16	− 9	Slightly enlarged; surface slightly nodular.	—	Spleen: 8 Retrogastric lymph node: 5 Lung: 8 Liver: 2 Kidney: 1 Testicle: 0	+
21	"	Subcutaneous.	22	+17	Slightly enlarged; surface nodular.	Minute, scattering, refractile tubercles in liver.	Spleen: 150 Regional lymph node: countless. Kidney: 90 Lung: 8 Testicle: 1 Liver: 1	+

22	Female.	Intraperitoneal.	22	+53	Slightly swollen; congestion; surface nodular.	A few minute scattering tubercles.	Spleen: countless. Retrogastric lymph node: 70 Kidney: 100 Liver: 5 Lung: 5 Uterus: 0	+
23	"	"	44	+153	Somewhat enlarged; congested; nodular surface.	Minute grayish necrotic foci in liver; few small tubercles.	Spleen: 35 Retrogastric lymph nodes: 10 Lung: 2 Uterus: 1 Kidney: 1 Liver: 0	+
24	Male.	Subcutaneous.	44	+125	Twice normal size; congested and very dark; nodular surface.	Fibrous induration of right testicle; disintegration of epididymis.	Spleen: 70 Regional lymph nodes: 10 Testicle: 1 Liver: 1 Lung: contaminated.	+
25	"	Intraperitoneal.	49	+151	Twice normal size; congested; prominent nodules on surface.	Minute necrotic foci in liver; large colon adherent in sublumbar region; abscess at adherent point.	Spleen: 30 Liver: 4 Lung: 1 Kidney: 1 Testicle: 1	+
26	Female.	Subcutaneous.	49	+98	Six times normal size; congested; smooth, easily torn surface.	Minute yellowish gray necrotic foci in liver.	Spleen: 45 Regional lymph node: 35 Liver: 1 Kidney: 1 Lung: contaminated.	+

TABLE III—*Concluded.*

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.		Result.
			days	*gm.*					
27	Female.	Intraperitoneal.	28	+47	Twice normal size; surface nodular.	A few minute tubercles in liver.	Spleen: Retrogastric lymph node: Liver: Uterus: Kidney: many. Lung: contaminated.	250 80 23 1	+
28	Male.	Subcutaneous.	29	+11	Slightly enlarged; congested; surface nodular.	Minute necrotic foci in liver; a few scattering refractile tubercles; subcutaneous abscess at site of injection.	Spleen: Regional lymph node: Liver: Kidney: Lung: contaminated.	150 125 2 2	+
29	"	Intraperitoneal.	38	+99	Slightly enlarged; congested; nodular surface; fibrosis of the center.	Omentum adherent to peritoneum in two places.	Spleen: Retrogastric lymph node: Kidney: Lung: Liver: Testicle:	90 75 4 1 0 0	+
30	Female.	Subcutaneous.	38	+125	Eight times normal size; congested; surface and borders nodular.	Scattering tubercles in liver; large colon adherent to dorsal wall.	Spleen: Regional lymph node: Kidney: Lung: Liver: Uterus:	35 15 8 7 0 0	+

highest toward the 4th week. In animals killed later the number appears to diminish gradually. On the other hand, the lesions manifest to the naked eye appear to become more conspicuous and widespread as the number of bacteria tends to decline. One might therefore venture the general statement that for a diagnosis based on the isolation of *Bacillus abortus*, guinea pigs should be killed between the 3rd and the 4th week. For a diagnosis based on characteristic lesions they should be killed later, preferably after 7 or 8 weeks. These statements do not hold rigidly, since the progress of the disease depends largely on the dosage of the virus injected and cases have been found in this laboratory in which extensive lesions were found within 4 weeks. Table IV shows that in most instances the spleens become quite large in 4 weeks.

Series IV.

The material used in this series was from several different sources. The inoculated guinea pigs were handed over to me by Dr. Smith, who had already obtained cultures of *Bacillus abortus* directly from the material, and who inferred, therefore, that most if not all of the guinea pigs would yield positive cultures. The material came from six different cases and included fifteen guinea pigs (Table IV). They were autopsied in the 5th week. The fetal membranes of Cows 203 and 210 came from presumably full time calves. Fetuses 205a and 205b were twins from Cow 205 from which amniotic fluid had been collected at the time of delivery. Fetus 206 was of uncertain age, 28 inches long and probably between 7 and 8 months old.

The negative outcome of inoculations of meconium as compared with the positive results of inoculations of the contents of the fourth stomach should be noted. I am informed by Dr. Smith that direct cultures from meconium of Fetus 205a were positive, but the colonies were very scarce. The same was true of cultures from meconium of Fetus 205b. In these instances, therefore, direct cultures from the fetus were more reliable than guinea pig inoculations.

Series V.

This lot of seven male guinea pigs was injected with gradually increasing dilutions of a fresh culture of *Bacillus abortus*. The object of

TABLE IV.

Results of Inoculation of Guinea Pigs with Fresh Material from Six Different Sources.

Guinea pig No.	Sex.	Source of material.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
				days	*gm.*				
31	Male.	Salt solution suspension (2 cc.) of meconium of Fetus 200.	Intraperitoneal.	31	+141	Slightly enlarged; nodular surface.	Minute necrotic foci in liver; hyperemia.	Spleen: 105 Liver: 0 Testicle: 0	+
32	"	1.5 cc. of thick salt solution suspension of 4th stomach contents of Fetus 200.	"	31	+106	Twice normal size surface quite nodular.	Fatty liver; necrosis of epididymis of both testicles; injection of vessels.	Spleen: 150 Testicle: countless. Liver: 0	+
33	"	0.3 cc. of salt solution suspension of cotyledon of Placenta 203.	Subcutaneous.	33	+111	Slightly enlarged; congested and dark.	Regional lymph nodes swollen; small abscess at injection site.	Spleen: 46 Regional lymph node: 75 Liver: 0	+
34	"	0.3 cc. of salt solution suspension of cotyledon of Placenta 203.	"	33	+165	Twice normal size; congested; faintly nodular.	—	Spleen: 90 Liver: 0 Testicle: 0	+

35	Male.	1 cc. of sterile amniotic fluid of Cow 205.	Subcutaneous	29	+181	Twice normal size; congested; surface nodular.	Minute scattering tubercles in liver.	Spleen: 50 Regional lymph node: 60 Testicle: 0 Liver: 0	+
36	"	2 cc. of salt solution suspension of amniotic fluid of Cow 205.	"	31	+109	Four times normal size; severe congestion; smooth, easily torn surface.	Minute scattering tubercles in liver.	Spleen: 70 Regional lymph node: 80 Liver: contaminated.	+
37	"	1 cc. of salt solution suspension of meconium of Fetus 205a.	Intraperitoneal.	29	+129	Normal.	—	Spleen: 0 Liver: 0 Testicle: 0	−
38	"	4th stomach contents of Fetus 205a 0.5 cc. + 0.5 cc. of salt solution.	"	30	+25	Twice normal size; surface nodular; nodules numerous.	Minute scattering tubercles in liver.	Spleen: 18 Testicle: 6 Liver: 0	+
39	"	4th stomach contents of Fetus 205b 0.5 cc. + 0.5 cc. of salt solution.	"	33	+80	Four times normal size; marked congestion; nodular surface.	Disintegration of epididymis of both testicles; vessels injected.	Spleen: 16 Testicle: countless. Liver: 0	+
40	"	1 cc. of salt solution suspension of meconium of Fetus 205b.	"	33	+139	Twice normal size; nodular surface.	—	Spleen: 0 Testicle: 0 Liver: 0	−
41	"	4th stomach contents of Fetus 206 in salt solution.	Subcutaneous.	30	+70	Three times normal size; surface nodular.	Minute necrotic foci in liver; testicles congested.	Spleen: 350 Testicle: 0 Liver: 0	+

TABLE IV—*Concluded.*

Guinea pig No.	Sex.	Source of material.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
				days	*gm.*				
42	Male.	1.5 cc. of salt solution suspension of lung of Fetus 206 ground with sand.	Subcutaneous.	30	+114	Three times normal size; markedly congested; easily torn.	Minute scattering tubercles in liver.	Spleen: 150 Liver: 2 Testicle: 0	+
43	"	0.6 cc. of salt solution suspension of cotyledon of Placenta 210.	"	34	+72	Five times normal size; congested; surface nodular; nodules prominent.	Minute tubercles in liver. Epididymis of both testicles disintegrated; abscess in left testicle; congestion.	Spleen: thin film. Liver: 150 Testicle: 2	+
44	"	0.3 cc. of salt solution suspension of cotyledon of Placenta 210.	"	34	+208	Eight times normal size; congested; distended capsule; easily torn.	Numerous minute tubercles in liver.	Spleen: 80 Liver: 1 Testicle: 0	+
45	"	0.5 cc. of salt solution suspension of cotyledon of Placenta 210.	"	34	+162	Five times normal size; congested; nodular surface; nodules numerous.	Few minute tubercles in liver.	Spleen: 70 Liver: 1 Testicle: countless. Regional lymph node: 17	+

the experiment was to determine whether or not there is a marked difference in the gross lesions produced, and in the number of organisms present, indicated by colonies found in cultures prepared from the spleens, in the guinea pigs injected with low dilutions and those injected with high dilutions. In other words, I wished to find any difference in the severity of the disease in guinea pigs inoculated with material heavily loaded with *Bacillus abortus*, and in those inoculated with mildly infected material, all animals being autopsied within the 4th week after injection.

The culture was obtained from Dr. Smith, who had recovered it from Cow 214, after passage through a guinea pig. A 72 hour agar slant was washed off with 2 cc. of sterile salt solution. After removal to a sterile tube the material was diluted to a comparative density of a 24 hour bouillon culture of *Bacillus typhosus*. 1 : 50 of this original dilution was injected into the first guinea pig, and the dilution doubled for each succeeding animal to the seventh, which received a dilution of 1 : 3,200. All the guinea pigs were injected into the peritoneal cavity. Table V is a summary of the results. Owing to an error, cultures prepared from organs of Guinea Pigs 50 and 52 were both labeled Guinea Pig 50; therefore a definite statement is impossible, but there was an average of about 100 colonies from the spleens of both guinea pigs.

The difference in the number of colonies present in the organs of inoculated guinea pigs, indicated by colonies counted on agar slants, is not marked between those receiving injections of heavy and light suspensions of the bacillus of abortion. It is of considerable significance, however, that positive cultures can be obtained from the organs between the 3rd and the 4th weeks after inoculation with material that harbors very few organisms.

Series VI.

In order to compare the relation of the lesions and growth of cultures prepared from inoculated guinea pigs autopsied 4 weeks after injection and those kept for 4 months, the three animals given in Table VI were chloroformed and examined. Guinea Pig 53 showed no characteristic lesions, and the bacterial count was low. Guinea Pigs 54

TABLE V.

Results of Inoculation of Guinea Pigs with a Pure Culture of B. abortus, Isolated through Guinea Pigs from Cow 214, the Number of Organisms Injected Being Gradually Diminished by Increasing the Dilution.

Guinea pig No.	Sex.	Dose injected.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
46	Male.	1 cc. of 1:50 the original dilution.	23	+	Possibly very slight increase in bulk; hyperemic; nodular surface.	Minute necrotic foci in liver; spermatic vessels injected.	Spleen: 170 Retrogastric lymph node: 100	+
47	"	1 cc. of 1:100 the original dilution.	23	+154	Twice normal bulk; mild congestion; surface nodular.	Few minute necrotic foci in liver.	Spleen: countless. Retrogastric lymph node: 48	+
48	"	1 cc. of 1:200 the original dilution.	23	+105	Hyperemic; no enlargement.	Few minute necrotic foci in liver.	Spleen: 50 Retrogastric lymph node: 15	+
49	"	1 cc. of 1:400 the original dilution.	23	+152	Twice normal bulk; congested.	Liver slightly enlarged; few minute necrotic foci. Spermatic vessels injected.	Spleen: 215 Retrogastric lymph node: 25	+
50	"	1 cc. of 1:800 the original dilution.	23	+167	Slight enlargement; hyperemic.	Two or three minute yellowish foci in liver; kidneys very dark.	Colonies present in all cultures.	+
51	"	1 cc. of 1:1,600 the original dilution.	23	+142	Normal.	Small intestine congested.	Spleen: 140 Retrogastric lymph node: 125	+
52	"	1 cc. of 1:3,200 the original dilution.	23	+92	Hyperemic.	A few minute yellowish areas in liver.	Colonies present in all cultures.	+

TABLE VI.

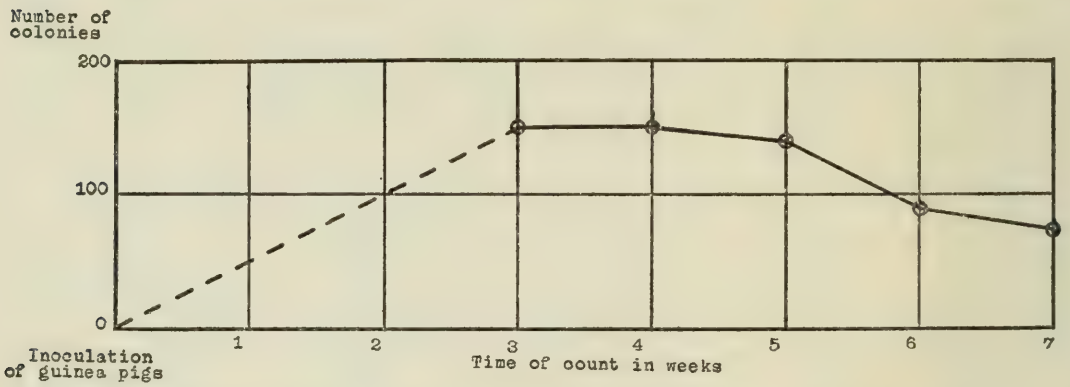
Results of Inoculation of Three Guinea Pigs with Fresh Material from Different Sources. Autopsies Performed about 4 Months after Injection.

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.		Result.
			days	*gm.*					
53	Male.	Intraperitoneal.	123	+293	Twice normal bulk; hyperemic; surface faintly nodular.	Liver dark; few small circumscribed tubercles. Vessels supplying testicles injected.	Spleen: Liver: Testicle: Kidney:	16 1 0 0	+
54	"	"	118	+215	Bulk increased at least ten times; very dark; surface nodular and easily torn.	Few minute tubercles in liver. Induration of epididymis of both testicles; small necrotic center to the mass.	Spleen: Liver: Testicle: Kidney:	45 2 0 0	+
55	"	"	110	+291	Bulk increased at least ten times; severe congestion; surface nodular; capsule easily torn.	Few minute circumscribed tubercles in liver. Atrophy of testicles; induration of epididymis; vessels injected.	Spleen: Liver: Testicle: Kidney:	90 2 2 0	+

and 55 presented the characteristic enormously enlarged, nodular spleen, atrophy of the testicles, and induration of the epididymis. In all three the number of colonies, counted in agar slant cultures of the spleen, was less than those counted on cultures prepared from guinea pigs autopsied between the 3rd and 4th weeks after inoculation.

Analysis of Results.

The foregoing experiments tend to confirm earlier work in demonstrating an inoculation disease in guinea pigs due to *Bacillus abortus* of Bang which is regularly associated with an enlarged, congested spleen. Other less constant lesions affect the testicles, kidneys, and bones.



The broken line indicates no count made; the solid portion indicates the beginning of the count.

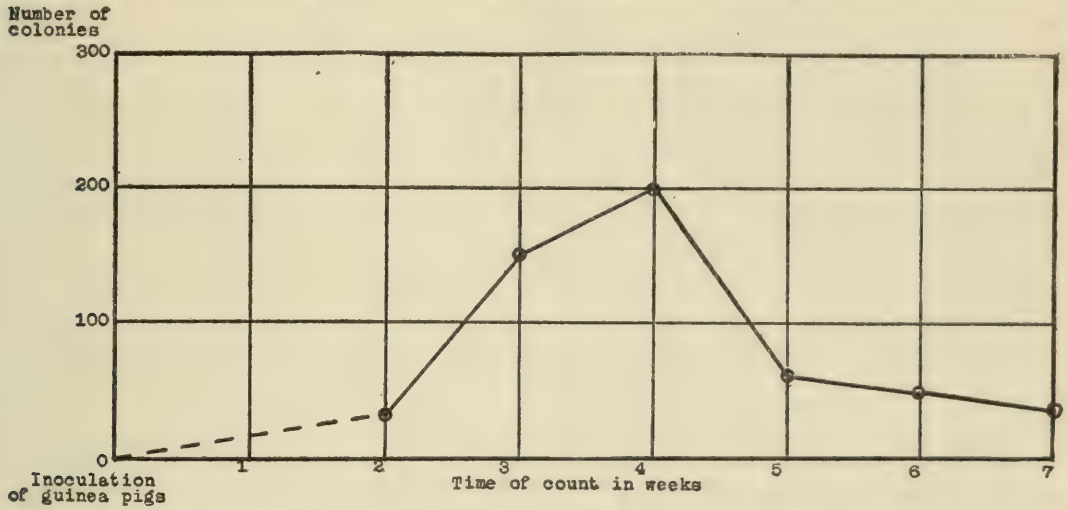
TEXT-FIG. 1. Guinea pigs of Series I inoculated with *B. abortus* 86. Colonies counted in cultures from spleens.

Minute foci frequently occur in the liver. There is, as a rule, no local lesion after subcutaneous inoculation. The animal regularly gains in weight. There is no appreciable difference in the result, whether the infection is introduced subcutaneously or into the abdomen. Nor does the size of the infecting dose within certain limits affect the result. Amounts varying between a standard dose and one-sixty-fourth of the same produced nearly the same results.

The immediate object of the investigation was to determine how far the incubation period in the guinea pig could be shortened. The results given above and Text-figs. 1 and 2 show that cultures of *Bacillus abortus* are regularly recovered from inoculated guinea pigs within

3 to 4 weeks. The figures obtained show that the number of living bacteria in the spleen of the guinea pig is larger at that time than later, although the macroscopic lesions tend to become more prominent as the bacteria decline. It remains to be seen whether the period cannot be shortened still more.

At least two or three culture tubes should be inoculated with bits of spleen tissue. In the series described such cultures were successful in 53 out of 55 cases. The two negative inoculations came from guinea pigs not affected with the disease; *i.e.*, they did not receive *Bacillus abortus* in the material inoculated. For diagnostic purposes there is no need of inoculating tubes from organs other than the spleen.



TEXT-FIG. 2. Guinea pigs of Series III inoculated with Placenta 146. Colonies counted in cultures from spleens.

CONCLUSIONS.

1. *Bacillus abortus* Bang can be regularly recovered from guinea pigs inoculated with material containing the bacillus within 3 to 4 weeks.

2. The method is especially useful in recovering the organism from fetal membranes which, as a rule, are obtained after having come in contact with fecal matter, bedding, etc.

3. The spleen is the organ in which the bacteria are regularly present and in largest numbers. Cultures must be made from it to ensure success.

[Reprinted from The Journal of Experimental Medicine, November 1,1918,
Vol. xxviii, No. 5, pp. 623–628.

A NEW TYPE OF SYRINGE, ESPECIALLY ADAPTED FOR INTRAVENOUS INJECTIONS OR THE ASPIRATION OF BLOOD.

By JAMES HOWARD BROWN, Ph.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

Plates 61 to 63.

(Received for publication, September 4, 1918.)

During the present war The Rockefeller Institute resumed the production of antimeningococcus serum and increased its output of antipneumococcus serum. At the entrance of the United States into the struggle the Institute undertook the production of these sera in much larger quantity than previously. The immunization of a large number of horses introduced many technical problems which were of relatively little importance in the immunization of only a few animals. Among these problems was that of a satisfactory syringe.

In the immunization of horses against meningococci and pneumococci fresh living cultures are injected intravenously. The amount of material injected is usually from 15 to 30 cc. For this purpose syringes made entirely of glass have been used. Their desirable features are (1) simplicity and the ease with which they may be cleaned, (2) the convenient slip joint for attaching the needle, and (3) the smoothly running ground glass plunger enabling one to sense by the pressure whether the material is being injected into or outside the vein. The objections to syringes of this type are (1) their perishability as a result of accident or of frequent boiling, (2) the difficulty of dislodging foam or bubbles after drawing up material into the syringe and the consequent danger of injecting air, and (3) the liability to lose material or to draw in air because of the ease with which the plunger moves. The last objection does not apply to the small syringes made entirely of glass but the weight of the contents or of the plunger in the larger syringes (25 cc.) is sufficient to move the plunger against the friction

of the binding spring supplied with these syringes. The conditions are not ideal when one is approaching a nervous horse.

Because of these difficulties there was much loss of time, breakage of syringes, and inaccuracy of dosage—difficulties which were serious for the work in hand and of constant occurrence. Catalogues of manufacturers and supply houses were searched and many kinds of syringes were tried; others could not be obtained readily because of war conditions. Many were too intricate, difficult to clean, to keep in repair, or easily broken, and expensive to replace. Those with expanding rubber or leather plungers worked hard, jumped, or leaked. None was so satisfactory as the syringe made entirely of glass in use.

The syringe to be described was designed and many of them have now been in use for over 6 months with good results. The barrel consists of an ordinary 50 cc. centrifuge tube (Fig. 1, *A*) or large test-tube (inside diameter $\frac{15}{16}$ to 1 inch). This is the only breakable part of the syringe. The plunger is made of metal (nickeled brass) with expanding rubber packing (Fig. 1, *D*). The outlet is through the plunger instead of at the end of the barrel. The plunger is made up as follows:

There is a core of metal tubing $6\frac{1}{2}$ inches in length (Fig. 1, *D*) with a bore $\frac{1}{16}$ inch in diameter. The upper end of the core is cut to fit a Luer needle (Fig. 1, *B*). The lower end terminates in a piece of hemispherical metal $\frac{7}{8}$ inch in diameter (Fig. 1, *D*) of suitable size to fit the bottom of the glass tube (Fig. 1, *A*). The bore of the core does not continue straight through the hemispherical lower end but communicates with a hole of equal size leading laterally from near the edge of the hemisphere (Fig. 1, *Dd*). Over the core and next to the hemispherical lower end is fitted a thick rubber washer (cut from pressure tubing, outside diameter $\frac{7}{16}$ inch), then a thick metal washer (outside diameter $\frac{5}{8}$ inch), and finally another rubber washer. The packing consists of a piece of pure gum rubber tubing (outside diameter $\frac{7}{8}$ inch, inside diameter $\frac{5}{8}$ inch) (Fig. 1, *C*) with smoothly cut edges and of a length slightly less than the total thickness of the series of washers on the core (about $\frac{5}{8}$ inch). It is essential to cut the packing from a piece of tubing with a perfectly smooth outer surface. Linen-finished tubing or tubing with a little longitudinal furrow in the line of the seam is not suitable. The packing is placed over the core around the series of washers and is centered by the metal washer. The lower edge of the packing rests against the hemisphere and the upper edge against the sleeve (Fig. 1, *F*) which is slipped on over the core after the packing is in place. Screwed onto the core above the sleeve is a wing nut (Fig. 1, *E*). As this nut is screwed down against the sleeve it causes the latter to expand the packing. There is formed between the rubber washers and the packing an air chamber the function of which will soon be described.

In use the plungers assembled as described are sterilized by boiling. The dose of material for injection is measured out by pipette into the cotton-stoppered sterile glass tube (Fig. 1, *A*). The cotton plug is discarded and the plunger is inserted into the tube (Fig. 2). The upper end of the tube is sealed by a rubber stopper with a metal bore which fits loosely around the sleeve of the plunger (Fig. 1, *Fa*). While the plunger is held by one of the milled areas around the sleeve the wing nut is screwed down until the expanding packing makes contact with the sides of the glass tube. Held in the position shown in Fig. 2 with the outlet in the hemispherical end of the plunger uppermost, bubbles of air or froth are easily expelled; hence, the reason for placing this outlet at the side rather than at the center of the hemisphere. If it is desired to leave a few small bubbles in the syringe rather than lose the small amount of material which may accompany their expulsion, they may be left in the syringe with perfect safety if during injection the syringe is turned so that the outlet in the side of the hemisphere is at the lower side of the tube; in this position the bubbles remain at the upper side and do not find the outlet. Injection may be made at any angle. In the intravenous injection of horses at this Institute it is customary to inject upwards. The needle (detached) is first inserted upwards into the jugular vein, the thumb being pressed over the jugular below the needle to cause the vein to bulge. When a small stream of blood coming through the needle shows that it is in the vein, the syringe is connected with the needle and the material injected by pushing the tube or barrel of the syringe against the plunger. The motion required is the same as that employed in pushing the plunger into the barrel of the ordinary syringe. The wing nut serves also as a finger rest (Fig. 2).

The function of the air chamber within the packing remains to be described. As mentioned above the ease with which the ground glass plungers of the syringes made entirely of glass move in the barrel is of great advantage in enabling the operator to detect by sense of touch any increase of resistance to the outflow of the material, often due to the slipping of the needle out of the vein during the injection of a restless horse. On the other hand, the rubber plungers of syringes on the market were found to work hard or jump if expanded sufficiently to prevent their leaking. With the syringe

being described the same difficulty was encountered until it was discovered that it could be remedied by leaving an air chamber within the packing and communicating freely with the interior of the syringe by a single small hole (Fig. 1, *Dc*) which runs diagonally upward through the edge of the hemisphere from a point near the outlet. The capacity of the air chamber in the syringes being used is about $\frac{1}{15}$ cubic inch. At first thought it might seem that injection material would pass into the air chamber and be wasted, but such is not the case. When during injection pressure is exerted on the bottom of the syringe tube a very small amount of material may pass into the air chamber but in so doing it compresses the air within the chamber and as soon as this pressure is relieved, as it invariably is towards the end of the injection, the drop of material is again forced out of the chamber by the expanding air. The compression of the air within the chamber serves another purpose; it causes the packing to expand automatically in response to pressure of all degrees. All that is necessary is to turn the wing nut sufficiently to cause the packing to make contact with the interior of the glass tube on all sides. In this condition the plunger moves easily and smoothly. As soon as the resistance to outflow increases, however, the packing expands and always just sufficiently to prevent leakage by the packing. In fact one may place the tip of the syringe against a rubber stopper on a table or against the wall and bear his whole weight against the bottom of the glass tube and the contents will not leak by the plunger, but if the resistance to outflow is removed the contents are expelled with the greatest ease. The ease with which the plunger moves is comparable only with that of a ground glass or metal plunger.

The syringe as above described is best adapted for injection, though it may also be used for aspiration against slight resistance. For aspiration against heavy resistance the arrangement of washers should be as shown in Fig. 3. With this arrangement the air chamber communicates with the atmospheric air through a small opening (Fig. 3, *c*) in the sleeve (closed by the upper rubber washer in Fig. 1).

If the syringe is desired to serve for both injection and aspiration against heavy resistance, it may be assembled as shown in Fig. 4, with two air chambers, the upper one expanding during aspiration and the lower one during injection.

The advantages claimed for the syringe are especially those desired for the intravenous injection of horses in large numbers where economy of time and expense, and the elimination of error are of great importance. These advantages are: (1) durability—non-breakage of expensive parts, the use of glass tubes for barrels, the use of rubber tubing for packing; (2) an easily moving, non-leakable, automatically expanding plunger; (3) the easy expulsion of air bubbles and froth; (4) the ability to measure out the doses and fill the syringes in the laboratory rather than in the stables.

As used in this Institute the syringes are filled in the laboratory, placed in the sterilizers in which the plungers have been boiled, and sent to the stables. At the stables each horse which is to be injected is turned about in the stall, tied with head outwards towards the aisle. The veterinarian with syringes, needles, absorbent cotton, and alcohol on a ward carriage moves down the aisle injecting the horses as he comes to them. The time required averages less than 2 minutes for each horse. A separate syringe is used for each horse, but it is suggested that it is possible to inject a series of animals, receiving the same material, with the same syringe plunger. The doses may be measured out into a series of the glass tubes, and the plunger moved from one tube to another for each injection. The cost of the syringe is moderate, the metal parts of those specially made for us costing less than $4.00 each.[1] The rubber packing of syringes in daily use needs to be renewed once in about 3 months. During the past 6 months over 3,000 injections have been made without serious accident due to defect of syringes.

It is suggested that there may be other uses for this type of syringe. Blood cultures might be made by aspirating blood into the tube of the syringe containing bouillon, the plunger removed, a sterile cotton plug inserted, and the tube incubated. For the collection of blood corpuscles blood might be aspirated into the tube of the syringe containing citrate solution, the plunger removed, a sterile stopper inserted,

[1] The syringes used have been ordered through George Tiemann and Company, 107 East 28th Street, New York City, and were made by Becton, Dickinson and Company, Rutherford, N. J. Suitable tubing for rubber packing has been obtained from the Scientific Materials Company, Pittsburg, Pa.

and the tube placed into the centrifuge. A slight variation of the technique would enable one to collect small samples of serum or defibrinated blood aseptically.

EXPLANATION OF PLATES.

PLATE 61.

FIG. 1. (*A*) Centrifuge tube used for barrel of syringe.

(*B*) Luer needle.

(*C*) Rubber packing cut from tubing.

(*D*) Core of plunger. (*a*) Rubber washers. (*b*) Metal washer. (*c*) Opening into air chamber. (*d*) Outlet communicating with bore of plunger.

(*E*) Wing nut and finger rest.

(*F*) Sleeve to be placed over core above packing. (*a*) Metal-lined rubber stopper for closing open end of barrel.

PLATE 62.

FIG. 2. Assembled syringe held in position for expelling air, or for injection after the air has been expelled.

PLATE 63.

FIG. 3. Arrangement of washers and packing for aspiration. (*a*) Rubber washers. (*b*) Metal washers. (*c*) Opening into air chamber.

FIG. 4. Arrangement of washers and packing for both injection and aspiration. (*a*) Rubber washers. (*b*) Metal washers. (*c*) Openings into air chambers.

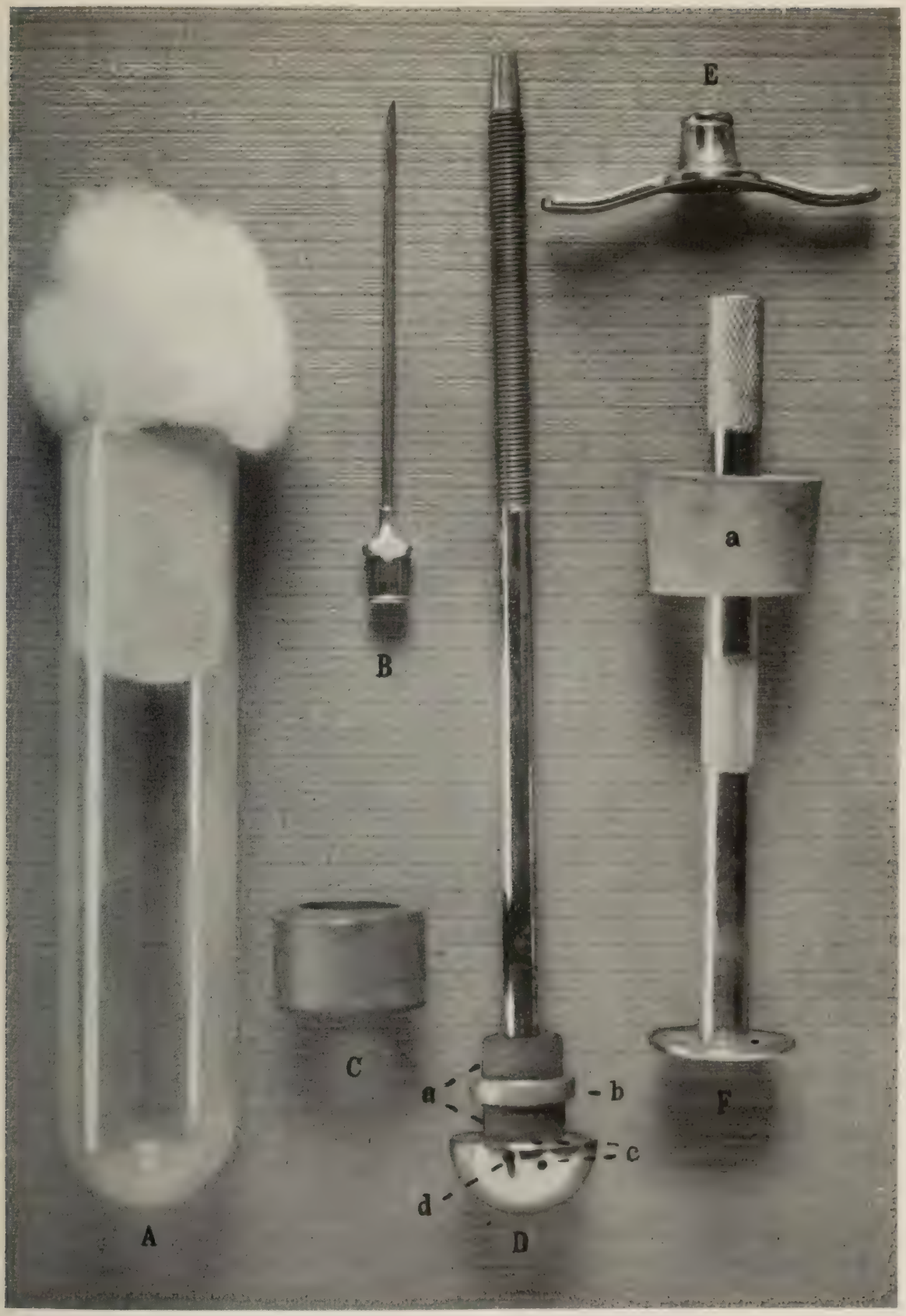


FIG. 1.

(Brown: New type of syringe.)

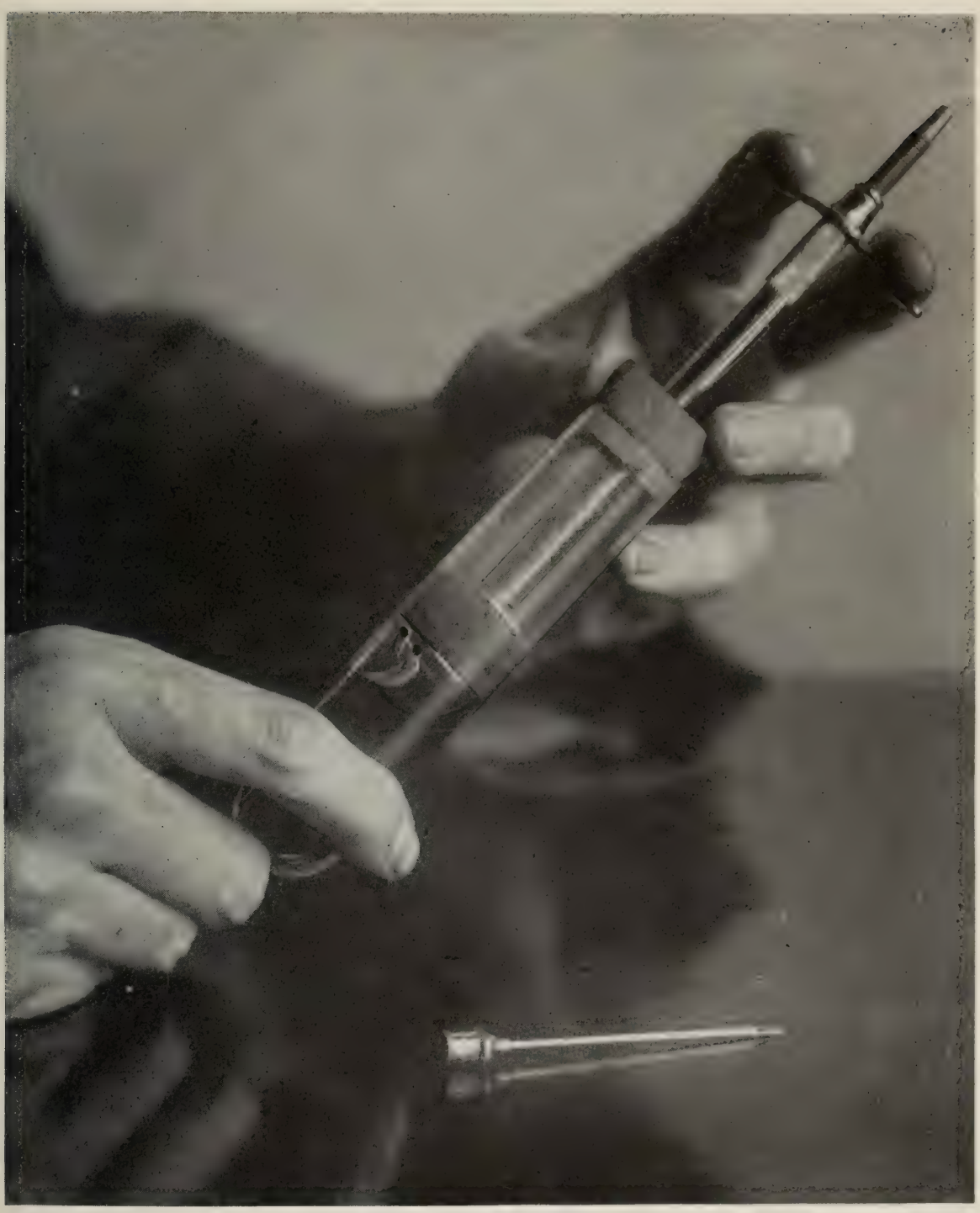


FIG. 2.

(Brown: New type of syringe.)

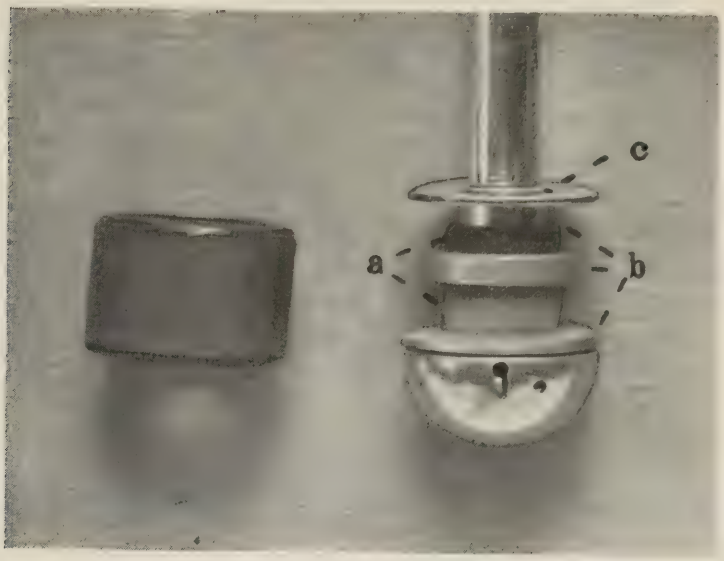


FIG. 3.

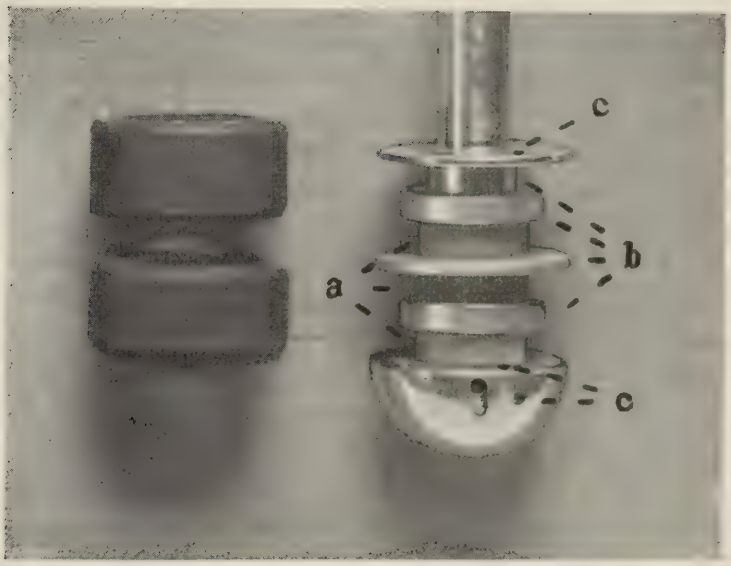


FIG. 4.

(Brown: New type of syringe.)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, November 1, 1918,
Vol. xxviii, No. 5, pp. 659–662.]

A RAPID DIFFERENTIAL METHOD FOR THE ISOLATION OF BACILLUS INFLUENZÆ.

BY JAMES HOWARD BROWN, PH.D., AND MARION L. ORCUTT.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

PLATE 77.

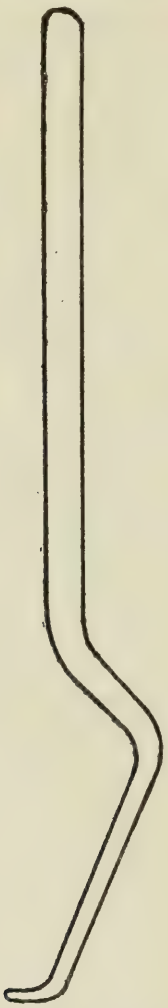
(Received for publication, October 1, 1918.)

It has often been noted that on a blood agar plate seeded with influenzal sputum the colonies of *Bacillus influenzæ* are most numerous and grow to larger size in the neighborhood of colonies of certain other organisms, notably the staphylococcus. This phenomenon led the authors to conduct various experiments which may be summarized as follows: Blood agar plates were seeded uniformly with a pure culture of *Bacillus influenzæ* and were then streaked with hemolytic and non-hemolytic staphylococci, hemolytic and non-hemolytic streptococci, and hemolytic and non-hemolytic strains of *Micrococcus catarrhalis*. After incubation it was found that the more luxuriant growth of *Bacillus influenzæ* occurred only in the neighborhood of the streaks of hemolytic staphylococci, streptococci, and micrococci, not in the neighborhood of the non-hemolytic strains. On laked blood agar the colonies of *Bacillus influenzæ* grew well throughout the plate and were no more numerous in the vicinity of staphylococcus, streptococcus, or micrococcus streaks than elsewhere. It has been shown[1] that the well known zones of hemolysis produced by hemolytic streptococci in blood agar are largely if not entirely the result of mere laking of the blood corpuscles and outward diffusion of the released hemoglobin into the surrounding medium. We would conclude, therefore, that the more luxuriant growth of *Bacillus influenzæ* in the neighborhood of colonies of hemolytic cocci is the

[1] Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, (in press).

result of the presence of released hemoglobin diffusing outward from those colonies. The hemoglobinophilic habit of *Bacillus influenzæ* has long been recognized as one of its differential characters.

The phenomenon described above has been utilized by us for the isolation of *Bacillus influenzæ* from sputum. To tubes containing about 12 cc. of standard meat infusion agar, melted and cooled to



TEXT-FIG. 1. Glass rod used for spreading droplets of sputum over the blood agar plate.

45° to 50°C., is added 5 to 10 per cent of defibrinated blood. In the fluid condition this medium is inoculated with a small loop of suitably diluted washed sputum and is immediately poured into a Petri dish. When the blood agar has solidified a very small loop of undiluted washed sputum is deposited on the surface of the medium

and by means of the flamed glass rod shown in Text-fig. 1 the droplet of sputum is smeared uniformly over the surface. Without flaming, the rod is then similarly rubbed over the surface of a second blood agar plate on which no sputum has been deposited by the loop. The optimum distribution of colonies should thus be secured on one or the other of the two plates. After the plates have thus been inoculated with sputum, each of them is streaked, in the form of a single streak, circle, or cross, with a pure culture of markedly hemolytic staphylococcus or streptococcus. After the moisture which collects on the surface of the freshly poured agar has been allowed to evaporate, the plates are incubated and may be examined next day. *Bacillus influenzæ* grows in the form of minute discrete convex colonies without hemolysis or discoloration of the blood agar and often visible only by reflected light. If colonies of *Bacillus influenzæ* are present they will be found in largest numbers and of largest size in the vicinity of the zone of hemolysis produced by the streak of staphylococcus or streptococcus. Material from such colonies should be stained by Gram's method, an aqueous solution of safranine being a good counterstain for *Bacillus influenzæ*. This method enables one to isolate within 24 hours Gram-negative hemoglobinophilic bacilli which, if they resemble *Bacillus influenzæ* morphologically and if the material has come from a case of clinical influenza, may be regarded as *Bacillus influenzæ*. Horse, human, or rabbit blood may be used, but to be of differential value it should be in good condition, not laked.

In Fig. 1 is shown the photograph of a plate inoculated as described above. In this sputum the principal organisms were hemolytic streptococci, shown as deep hemolytic colonies scattered throughout the plate, and influenza bacilli. The plate was streaked in the form of a circle by a stock strain of hemolytic streptococcus. The small refractive colonies of *Bacillus influenzæ* (indicated by arrows) are seen to be very numerous within the zones of hemolysis produced by the deep colonies of streptococci, also in the neighborhood of the streak of stock streptococcus, and actually within the streak itself. By reflected light they could also be seen in smaller number and of smaller size in portions of the plate remote from the zones of hemolysis but these colonies cannot be seen in the photograph.

EXPLANATION OF PLATE 77.

FIG. 1. Blood agar plate inoculated deeply and over the entire surface with influenzal sputum, and then streaked in the form of a circle with a stock strain of hemolytic streptococcus. Small discrete colonies of *B. influenzæ* are indicated by arrows.

FIG. 1.

(Brown and Orcutt: Rapid method for isolation of *B. influenzæ*.)

[Reprinted from The Journal of Experimental Medicine, December 1, 1918,
Vol. xxviii, No. 6, pp. 701–719.]

SPIRILLA ASSOCIATED WITH DISEASE OF THE FETAL MEMBRANES IN CATTLE (INFECTIOUS ABORTION).

By THEOBALD SMITH, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

Plates 81 and 82.

(Received for publication, September 24, 1918.)

In the course of investigations into the pathology and etiology of abortion in cattle as it prevailed among the animals composing a certain group of herds under one management, and with more or less intercourse between individual herds, there was encountered a fair number of cases from which *Bacillus abortus* Bang could not be isolated from the fetus or placenta either in cultures or through guinea pigs or with both methods combined. These cases ranged themselves into several groups and comprised (*a*) the few fetuses yielding sterile cultures, (*b*) those yielding rapidly growing cultures of colon-like and other bacteria, and (*c*) those yielding pure cultures of a spirillum. It is this latter organism which will be dealt with in this paper.

After much of the work here reported had been done, a report of an English Committee on abortion in sheep came to the writer's notice.[1] The actual work is credited to Sir J. McFadyean and Sir Stewart Stockman. The direction of the investigation was in the hands of a larger committee of which the two authors mentioned were members. The brief report of this committee summarizes the results of the experiments reported in full in the appendix.

The microorganism is described as a vibrio and the more precise nomenclature left for future determination. The vibrio was cultivated on the well known medium consisting of agar, gelatin, and serum in deep layer as first used by Bang[2] for *B. abortus*, and it grew in much the same way as this bacillus. A careful reading of the statements concerning the cultural characters of the

[1] Great Britain Board of Agriculture and Fisheries, Report of the departmental committee on epizootic abortion, London, 1913, 22.

[2] Bang, B., *Z. Thiermed.*, 1897, i, 241.

vibrio does not reveal any essential differences between this organism and the spirillum under discussion. Nor do we find any differences in their negative effects on small laboratory animals. Seven head of cattle were infected with exudate from aborting ewes either with or without the addition of pure cultures to the pathologic material. In two instances the mode of introducing the virus was by mouth, in the rest by intravenous inoculation. Two cases aborted, and in one of these the vibrios were detected. These results are as yet too meager and indefinite to answer the important question whether the vibrio of bovine abortion is the same as the spirillum of the bovine disease.

What is of more interest at the moment is a statement, quite buried in the report, of the isolation of vibrios from cases of abortion in cattle in a herd in Ireland and in Wales in 1911. A brief paragraph is all that is given of these findings and we are left to conjecture whether vibrios were found later in other herds.

Lesions Associated with the Spirillum.

In all, fourteen cases have come under our observation from which pure cultures of the spirillum have been obtained. At the same time the abortion bacillus of Bang was not met with in any of these cases.

Taking the cases consecutively as they occurred in the group of herds under observation since the earliest isolation of the spirillum, beginning June, 1917, and ending September, 1918, there were twenty-seven cases from which the bacillus of Bang was isolated and fourteen cases associated with spirilla. These figures are not to be considered accurate for the reason that a certain small number of cases were not studied and hence are not included. A certain number could not be studied so thoroughly as the rest and hence the associated organism may have been overlooked. Nevertheless, the figures may be taken as indicating that, in this group of animals as elsewhere, *Bacillus abortus* is at present the predominating organism in infections of the fetal membranes of cattle.

The cases examined indicate that the lesions associated with this organism are largely if not exclusively restricted to the fetal membranes and that the fetus suffers secondarily from a gradually increasing interference with the placental circulation. This is precisely what occurs when *Bacillus abortus* invades the chorion and cotyledons. We would therefore expect the changes in the fetus to be much the same in both etiological types. This is the case. It is impossible to

foretell whether a given fetus will yield cultures of *Bacillus abortus*, or of the spirillum, or none at all.

Unfortunately, the fetal membranes are either retained and discharged in a disintegrated and decomposed state, or if discharged with or soon after the fetus they are mostly in more or less advanced stages of autolysis, the death of the fetus having taken place some time previously. The material, therefore, which is the primary seat of the disease will be available in the best state only when cattle are slaughtered and the membranes examined fresh, *in situ*. Such cases are relatively rare and perhaps encountered only by accident. It may be that serological tests will enable us to select specifically affected animals during life.

The main lesions affecting the fetus are edema of the subcutaneous tissue and effusions into the large serous cavities. The fluids are, as a rule, more or less heavily tinged with blood. This effusion is frequently associated with delicate, loose, shreddy deposits of fibrin or more rarely heavy, whitish pseudomembranes in abdomen and much less abundantly on pleura and epicardium. The visceral changes are chiefly those referable to autolytic changes following death. Focal lesions are not present.

The stomachs usually contain considerable quantities of what appears to be swallowed meconium or perhaps, at least in some instances, material driven into the fourth stomach from the small intestines by antiperistaltic movements. The stomachs of normal fetuses contain a colorless, translucent, very thick, viscid fluid which in older fetuses may contain a few pellets of meconium and perhaps some hairs. The diseased fetus, however, almost invariably has in its stomachs a very turbid, thick, yellowish, flaky fluid. Not infrequently there are also found small, whitish, soft, disc-like masses which can be traced back to epithelial excrescences of the amnion and which have come away and been swallowed by the fetus with the amniotic fluid.

The lungs may or may not be inflated, depending on the age of the fetus. In some the air tubes contain fluid identical with that in the stomachs. This fluid may completely fill the trachea and bronchi.

The kidneys are usually surrounded by a thick bloody fluid which collects in a thin layer under the capsule and separates this from the cortex. The tissue about the kidneys is also frequently distended with fluid suffused with blood-coloring matter.

The study of sections of fixed and hardened tissues of fetuses has not brought out anything characteristic of the infection. In the digestive tract the epithelial coverings may be partly or wholly desquamated, probably not as the result of any disease process, but following death of the fetus. Focal lesions were absent here and from the other organs. The lungs in some cases presented collections of cells of undetermined type in the alveoli and smallest air tubes. This absence of active disease processes in the fetus is probably to be accounted for by facts to be submitted later on concerning the habitat of the spirillum.

About the fetal membranes, little that is definitely known can be said until cases are available in which the fetus and membranes can be studied fresh and *in situ*. In general, however, it may be stated that the focal lesions of the cotyledons and of the chorionic membrane between cotyledons, and the exudate, which characterize the cases infected with *Bacillus abortus*, are absent in the cases associated with spirilla. The histological picture resembles in certain respects only that of the disease due to *Bacillus abortus*. The chorionic epithelium is destroyed and the tissue immediately below it is densely infiltrated in spots with cells, whose nuclei are in a state of fragmentation, as if a strong chemotactic influence were at work in the utero-chorionic space. There is, furthermore, edema of the chorion and extensive, rather loose cell infiltration into the edematous zone. The nature of these cells, necrotic in the cases examined, has not been definitely made out. In several instances there were dense collections of polynuclear leucocytes among the denuded villi of the cotyledons.

Morphological and Biological Characters of the Spirilla.

The only microorganism thus far encountered in films and sections of the fourteen cases is a spirillum, if we exclude certain mixed infections of the fetus with *Bacillus coli*, miscellaneous bacteria

attaching themselves to the placenta as it is discharged into the bedding and manure, and those swallowed by the fetus over 7 months of age during and just after birth.

Spirilla have been detected microscopically both in the fetus and in the few placentas available, but not in every case from which pure cultures were obtained. If we grant that the seat of the disease is in the fetal membranes, more particularly the chorion, the spirilla make their way into the amniotic fluid and thence into the digestive and respiratory tracts of the fetus. Spirilla have been detected in films of stomach contents in five cases and in sections of the small intestine in two. In the placental tissues they have been seen either alone or with miscellaneous filth bacteria. Their precise habitat in the chorion cannot be fixed upon with the meager material available. In one case the endothelium of certain capillaries of the chorion was crowded with spirilla (Fig. 7). This condition has not been seen in the few other cases examined. In one other case the necrotic cores of the villi contained dense colonies of minute organisms which were probably spirilla. In several other cases spirilla were loosely scattered through the necrotic tissues. Inoculation of guinea pigs in the two latter instances showed that *Bacillus abortus* was absent. It should be stated that the short forms of the spirillum are very minute and when packed together in dense masses only those on the periphery may be recognizable.

In form the spirilla found in fixed and hardened tissues vary more or less in length. The short forms, consisting of 1 and $1\frac{1}{2}$ wave lengths or windings, predominate; those of 2 to 4 windings are less numerous. In the small intestines they occur in the desquamated epithelium and along the free margins of the villi (Fig. 1). There is evidence from the microscopic picture that still shorter forms exist which, owing to their minuteness, are not definable. The transverse diameter or thickness is probably not over 2 μ, the windings about 2 μ long, amplitude, 0.5 μ.

The importance of a suitable staining agent in disclosing these forms cannot be overestimated. Thus far alkaline methylene blue has given satisfactory results for films. Giemsa's stain, now on hand, has been less so. In fixed material eosin-methylene blue has been used with good results. Bacteria in tissues and fluids may be in

various stages of active multiplication and degeneration and of encapsulation. A given dye may therefore pick out only certain stages. This is probably true of methylene blue. Films of stomach fluid stained 24 to 48 hours in one case showed a dense tangle of long, rather feebly stained spirilla with and without stained granules (Fig. 2), whereas the 10 minute preparation showed only small numbers of fairly short, well stained forms. The spirillum is Gram-negative.

Spirilla from cultures do not present any features not found in the microorganisms in the host tissues and fluids. In the earliest growth the short forms predominate. As the culture grows older longer spirochete-like forms appear and become most numerous. At the same time, deeply stained granules may appear at spaced intervals in some of the spirilla. These are also found in individuals from body fluids and only in the long forms. These facts point towards degenerative processes but further studies may tend to contradict this view.

If bits of tissues or particles of meconium or drops of stomach contents containing spirilla are added to slanted agar and the tube is hermetically closed with sealing wax, there will be seen after 3 or 4 days, along the lateral margins of the slanted surface, between agar and glass, a narrow grayish line extending up from the condensation water one or several centimeters. At the same time or some days later a very thin, barely visible film of growth will have extended from this line inward between agar and glass. In some instances this film continues to spread until it meets the film growing in from the opposite side. Rarely a thin film starts upward on the slope for a few millimeters from the condensation water. If a transfer to a fresh tube of agar is made after 4 or 5 days, a similar though feeble growth may be obtained. A second transfer is, however, apt to fail.

In casting about for media suitable for the continued cultivation of the strains it was found that while tubes containing bits of guinea pig or other mammalian tissue are suitable, the best method was to add a few drops of defibrinated horse blood to the condensation water of ordinary nutrient agar slants. This medium has served the purpose very well and at the time of writing strains from twelve of

the fourteen cases from which spirilla have been isolated in pure culture are still alive. Some have been transferred over 60 times. In general the older strains grow more profusely. After the strains had been under cultivation for some time they were tried on plain agar, sealed, with the result that the oldest strains have grown and are continuing to multiply in the absence of blood or bits of tissue (Figs. 3 and 4).

The growth on blood agar varies somewhat from strain to strain but the main features are the same. In the older strains, multiplication occurs in a thin layer over the sedimented corpuscles in the condensation water, between glass and agar, as a grayish layer on the slope starting from the condensation water and as isolated colonies on the slope. The latter may be absent. When present, they vary in size from mere points to 2 mm. in diameter and have the ordinary appearance of bacterial colonies. On plain agar the growth is much less vigorous as a rule, but even in this medium occasionally the amount of growth is unexpectedly large. Quite invariably the growth in the condensation water resembles a drop of thick translucent mucus and, owing to its viscidity, it may be difficult to pull it away from the inside of the tube in transferring to a fresh tube. Growth also occurs between glass and agar and less frequently on the slope.

The usual cultural media, including carbohydrate media in fermentation tubes, have been tried without success. It is not improbable that after prolonged cultivation in sealed tubes of plain agar it may be possible to obtain cultures in bouillon, milk, etc.

In young cultures the spirilla are in active motion. The short forms, 1 to $1\frac{1}{2}$ windings long, move with great rapidity across the field of the microscope. The longer, more sluggish individuals of two to four windings move in a straight line, revolving about the longitudinal axis at the same time. The very long forms, often bent or curved, are very sluggish and move in irregular manner, about one end, or only revolve. Cultures more than a week old may contain no active individuals.

With this method of cultivation the spirilla have been isolated in pure culture, and quite uniformly so, from the fourth stomach, meconium, and lung tissue of the fetus. They were isolated not so

frequently from spleen, liver, and kidneys. In all cases bits of tissue as large as peas were transferred to the culture tube of plain agar. The invasion of the body generally is thus not the rule and may be due to accidentally favorable conditions.

The action of this organism on the small laboratory animals so far tried is apparently without harmful effects. Of the forty-odd guinea pigs inoculated subcutaneously and intraperitoneally with body fluids and tissues containing cultivable spirilla, all have thriven like normal animals. Several rabbits treated repeatedly with large doses injected into the abdominal cavity have remained well. No anaphylactic symptoms have developed. Rats and mice are also refractory to large doses of cultures. Birds have not yet been tested.

The resistance of the culture forms to spontaneous disintegration is evidently low. Cultures renewed after a period of a week may be lost. On the other hand, some resist. Thus of two original cultures from body tissues and fluids, one was still alive after $2\frac{1}{2}$ months. It was kept at room temperature and protected from drying by sealing wax. Another culture under the same conditions and only $3\frac{1}{2}$ weeks old was dead.

Owing to the time required to bring the various strains of spirilla to grow actively in media free from blood and tissues, serological tests to determine whether the strains react alike to immune sera are still under way and they will be described in a later publication. Preliminary tests with the serum of rabbits repeatedly inoculated with large doses of living spirilla indicate a close relationship of the fetal strains.

Probable Relation of the Spirillum to the Disease Process.

The proof of any direct etiological relation between a given microorganism and a definite disease process is brought by reproducing the latter with pure cultures of the microorganism in question. This should not be a difficult undertaking were it not that infectious abortion is widespread. To utilize for crucial tests animals from infected herds may lead to errors of interpretation in two directions. The inoculated animals may abort on account of spontaneous infection or they may have acquired more or less resistance and fail to

abort after inoculation. The experimental tests with the spirillum are therefore waiting for suitable subjects. In the meantime, the presumption that this microorganism bears a relation to abortion similar to that borne by *Bacillus abortus* is supported by several facts.

The spirillum is isolated in pure culture from fetuses under precisely the same conditions as is *Bacillus abortus*. The cultural peculiarities of the two in requiring a reduced oxygen tension are the same. The distribution of both organisms topographically is the same. Both occur quite regularly in the gastrointestinal and respiratory tracts of the fetus, more rarely in other viscera. Both occur in the placental fluids but *Bacillus abortus* is more readily demonstrable because of the susceptible guinea pig, which eliminates in itself the miscellaneous filth bacteria in the discharged fetal membranes.

The cases in which the spirillum was demonstrated did not harbor *Bacillus abortus*. None of the guinea pigs inoculated with fetal material or placenta has acquired the specific lesions produced by it. On the other hand, the spirillum was absent in the cases from which *Bacillus abortus* has been isolated. In the many hundreds of cultures prepared in this laboratory the bacillus of Bang develops after 5 to 10 days. This period gives the spirillum ample opportunity to multiply if present. No such mixed infection has been observed. It appears, therefore, that these organisms are mutually exclusive for reasons not known at present. A further significant fact which cannot be discussed until the cases associated with *Bacillus abortus* have been analyzed and presented is the limitation of the spirillum to second and later pregnancies thus far.

Another fact which supports the etiological rôle of the spirillum is the discovery of vibrios or spirilla in the abortion of sheep and in two instances in herds of cattle in Ireland and Wales, as has been stated above. Although the method of cultivation was different in our work from that used by the English workers, the evidence is sufficiently strong to warrant the inference that they are closely related.

TABLE I.

Cow No.	No. of sire.	Length of fetus.	Date of breeding.	Date of abortion.	No. of pregnancy after purchase.	Cultures.							Guinea pig inoculations.			
						4th stomach.	Meconium.	Lung.	Spleen.	Liver.	Kidney.	Fetal fluids.	4th stomach.	Lung.	Meconium.	Placenta.
		in.		*1918*												
67	?	6½	(Purchased Sept. 2, 1917.)	Jan. 6	?							+				−−
				1917												
90	?	22½	(Purchased Nov. 29, 1916.)	June 2	?	++	−−*			+						
			1916													
95	1	28½	Nov. 13	" 14	2nd			++								
			1917													
149	1	16½	June 10	Oct. 27	2nd	+		++					−	−−		
159	2	16¾	" 16	Nov. 7	3rd	Heavy growth.		+−	−		Several species.		−			−−
179	3	29	Apr. 23	" 26	2nd	+−			−		−		−	−		
				1918												
192		36	May 30	Jan. 23	3rd	?	++	+++	−−	−−	−−			−	−	
213	4, 5	21	Aug. 4 " 31	Mar. 18	4th	++		++	−−	−−	−−		−	−	−	
			1918													
246	2, 6	7½	Jan. 11 " 24	May 28	2nd			++−		++−				−	−†	
251	2	17½	" 12	June 10	2nd	++	++*	−+−					−	−		
256	6	19	" 14	" 18	4th	++		+	++	++	++		−	−		
			1917													
258	7	27	Dec. 16	" 29	2nd	?	++	+	++	++	++			−−	−−	
			1918													
263	3	20	Feb. 9	July 30	3rd	++	++	+++		++			−	−	−	
267	4	23	" 14	Aug. 12	3rd	++	++	++	++	++	++		−	−	−	

* Small intestine.

† Liver.

SUMMARY.

Spirilla of identical morphological and cultural characters have been isolated in pure culture from the fetuses of fourteen cases of abortion. The condition of the fetus is much the same whether spirilla or the bacilli of abortion are present. This condition is probably due in both cases to interference with the placental circulation. The injurious action of the etiological factor when spirilla are present is limited to the fetal membranes, more particularly the chorion. Definite lesions of the fetus were not detected. The spirilla gain access to the digestive and respiratory organs of the fetus when the latter swallows the amniotic fluid. More rarely they are disseminated through the body, probably through the circulation. The spirilla will grow in certain culture media only under reduced oxygen tension, readily secured by sealing the ordinary culture tubes with sealing wax. Laboratory animals (mammals) are refractory. The precise relation of the spirillum to the pathologic process remains to be more definitely formulated. Since the spirillum was first isolated, twenty-seven cases have been found associated with *Bacillus abortus* and fourteen with the spirillum. In none was a mixed infection with both organisms detected. The spirillum has been isolated only from the second or succeeding pregnancies.

Table I summarizes the data collected thus far. It gives by number the male and shows that the spirillum is not associated with any one bull. The spirillum has been found in fetuses of various ages as shown in Column 3. The distribution of spirilla as shown by cultures is given, the sign + indicating pure cultures, the sign − no growth. The guinea pig inoculations are shown to be uniformly negative as regards *Bacillus abortus*.

APPENDIX.

Inasmuch as no data have thus far been published concerning the occurrence of spirilla in bovine abortion it has seemed best to publish the following brief notes on the individual cases.

Cow 90.—Cow purchased November 29, 1916. Aborted June 2, 1917. Placenta discharged soon after. Both examined June 4 after having been refrigerated.

Male fetus, length 57 cm. Hairless, except on lips. Marked general subcutaneous edema with slight staining of fluid with hemoglobin. In abdomen and pleural and pericardial sacs considerable amounts of blood-tinged fluid together with thin elastic fibrinous deposits on liver, omentum, lungs, and heart. All organs are more or less soft, putty-like in consistency.

Stomachs contain a turbid, bile-stained, flaky viscid fluid. Cultures made by adding a few drops of stomach contents and a bit of liver to agar and sealing. In these, active spirilla found in pure culture.

Two guinea pigs inoculated with 1.3 cc. of a suspension in salt solution of cotyledons, ground in sand. Chloroformed after $2\frac{1}{2}$ months. No lesions of *B. abortus*. Cultures from spleen remain sterile.

Sections of the placenta fixed in Zenker's fluid and stained in eosin-methylene blue show a complete loss of the epithelium covering chorion and villi of cotyledons and the presence of groups of polynuclears at the roots of villi. In certain blood vessels of chorion from 10 to 120μ in diameter the endothelial cells are crowded with minute bacteria, not resembling *B. abortus* and in a situation where the latter is not found (Fig. 7). The minute organisms are probably short spirilla, for on the thin margin of groups, where they lie in single layer, short curved forms are recognizable. Endothelial cells within the lumina of these vessels are also crowded with the same forms. Where chorion and amnion are fused there is considerable edema with infiltration of cells no longer identifiable (necrotic).

Twin Fetuses 94 and 95.—Dropped on pasture June 14, 1917. Placenta was discharged some hours later but unfortunately was not brought to the laboratory with the fetuses.

Fetus 94.—Male fetus with good coat of hair, black and white; length 72 cm. No hemorrhagic discoloration or edema of subcutis. Mouth soiled with earth. Stomachs contain a thick, very viscid, dark greenish, opaque, flaky fluid. Liver large, rather pale; kidneys soft, with numerous cortical hemorrhages. Lungs indicate that some air has entered. Nothing noteworthy otherwise. Cultures from this fetus as follows: Those from spleen, kidney, and lungs remain free from growth. From this case no spirilla isolated. Sections from fixed tissues show nothing noteworthy beyond congestion.

Fetus 95.—Twin of No. 94. Abdominal organs, except spleen and liver, eaten out by some animal on pasture. Lungs contain a trifle air, but are otherwise heavy and full of blood.

Of five tubes prepared with bits of lung tissue, three developed into pure cultures of a spirillum. The two remaining contained spore-bearing aerobes.

Histological examination of the lung tissue shows presence in air tubes and alveoli of groups of cells of uncertain character. Interlobular tissue broadened and lymph spaces filled with blood.

Cow 149.—Cow aborted October 27, 1917. Due to calve March 19, 1918. Calved normally in 1916. Placenta now retained.

Fetus 16½ inches long, male. Skin readily pulled from underlying tissues exposing axillary and inguinal regions as if dissected by *B. welchii*. All muscles soft and readily torn away from attachments. Blood-tinted fluid in abdominal cavity. Liver macerated and tissue readily crushed. In the stomachs a pinkish fluid. The layer of epithelium detached and carried out by incision with the fluid like sheets of tissue paper.

Blood-tinted fluids in pleural sacs; lungs with slight interlobular edema. No odors of bacterial decomposition.

Pure cultures of a spirillum obtained from bits of lung tissue and stomach fluid. Cultures from other organs not made.

Two guinea pigs inoculated with suspensions of lung tissue and one with stomach contents. They were chloroformed after 7 weeks. No lesions found and all cultures from spleens remain sterile.

Cow 158.—This case is reported because it also was probably associated with spirilla, but cultures were not made. *B. abortus* was absent.

Cow purchased February 16, 1917. Calved normally March 10 and aborted November 6.

Fetus and membranes discharged together, the two still connected by umbilical cord. No odor of decomposition. Fetus 12½ inches long, female. In subcutis of ventral aspect and especially over pubis a translucent, only in part blood-tinted edema. Blood-tinted fluid in abdomen. Liver very friable. Thorax contains considerable blood-tinted fluid.

The placenta shows a glass-clear gelatinous edema between chorion and amnion. No liquid obtainable by incision into this layer. Cotyledons whitish; the villi long and slender when floated in water.

Three guinea pigs inoculated with 1 cc. of a turbid suspension of cotyledons ground up in salt solution were kept 8 weeks. They were then chloroformed and cultures prepared from bits of spleen tissue. All remained free from growth. The guinea pigs were normal.

Sections of fixed and hardened cotyledons show extensive collections of polynuclears between the villi denuded of epithelium. The villi are represented only by the necrotic connective tissue stroma. In this stroma there are exceedingly dense masses of minute bacteria which in places thin out and are resolved into short wavy forms.

Cow 159.—This cow was purchased in April, 1914. Calved normally in 1915. Aborted February 23, 1916 and November 7, 1917.

The fetus of 1916 was 3 to 4 inches long and discharged with membranes. It had undergone considerable maceration, the entire body being soft, semitranslucent and only head, limbs, and ribs recognizable. Odor peculiar but not suggestive of bacterial decomposition. Two guinea pigs inoculated with placental tissues remained well and were chloroformed after 10 weeks. No lesions were found and spleen cultures remained indefinitely free from growth. Sections of the placenta show groups of polynuclears among villi, but no spirilla among other bacteria.

The fetus and placenta of 1917 were obtained together. Fetus 16¾ inches long, male. Blood-tinted fluid in subcutis, in abdomen, perirenal tissue, thorax, and pericardial sac. A thin deposit of fibrin shreds on heart surface. Rumen contains a thick, viscid, pinkish fluid and a mass resembling the color and consistency of yolk of egg. Fourth stomach contains a similar fluid but no suspended matter.

The placenta received only in part. After removing shavings and other bedding, the cotyledons were found to be gray to whitish. There was no edema of the fetal membranes. The amniotic fluid still remaining was thin, slightly blood-tinted, with fine flakes in suspension.

Cultures were made from contents of fourth stomach and amniotic fluid. Only one from stomach contents showed growth in the form of a pure culture of spirilla.

Sections of fixed and hardened tissues of fetus presented nothing noteworthy. Spirilla were detected in contents of small intestine. Sections of the chorion, including portions of cotyledons, showed infiltration of cells into the wall of the small blood vessels. The cells are probably mononuclear but the bizarre form of the nuclei makes it impossible to define them. On the periphery of the cell infiltration are many curved forms, of one to two windings, very minute. They are not in dense groups but scattered singly through the tissue. None is seen in the endothelium of the vessels as is the case in No. 90. There is some fibrin in the vascular walls and also in the nearly nude villi of the cotyledons. Polynuclears are absent but this may be due to the washing of the fetal membranes necessitated by the filth attached to them. Miscellaneous bacteria are, as might be expected, attached to the free exposed surfaces of the fetal membranes.

Three guinea pigs were inoculated, one with suspension of ground up cotyledons, one with contents of fourth stomach, and one with amniotic fluid. After 8 weeks they were chloroformed. Cultures from the spleens remained free from growth.

Cow 179.—Cow aborted November 26, 1917. The fetus was placed in refrigerator and examined November 28. Placenta retained.

Fetus 74 cm. long, with a good coat of hair. Surface of body stained yellowish. Some shavings from bedding in mouth. Abdomen somewhat distended.

Stomachs filled with a yellowish, only slightly viscid fluid, holding in suspension abundant soft yellowish brown masses. Large intestine well distended with meconium. Liver slightly enlarged, yellowish. Spleen on section shows some lighter areas. Lungs not air-distended. Trachea and bronchi filled with stomach contents.

Cultures were made directly from fetal tissues. Three tubes inoculated respectively with contents of fourth stomach, spleen, and kidney tissue remained sterile. In one kidney and lung tube heavy growths appeared of several species. In one tube of stomach contents a pure culture of spirilla developed. In one liver tube a small capsulated bacillus appeared. Sections of various organs, fixed and hardened, show nothing characteristic or noteworthy.

Two guinea pigs inoculated, one with a suspension of ground up lung tissue, the other with stomach contents, were chloroformed after 7 weeks. Lesions absent. Cultures from spleen negative.

Cow 67.—Fetus, within membranes. Discharged January 6, 1918, and placed at once into refrigerator. Examined January 7.

Fetus about 17 cm. long. Head resting on abdomen and directed caudad. Cervical vertebræ separated and only skin and sternomastoid muscle holding head to body. Tissues soft and partly autolyzed. Allantoic and amniotic fluids turbid, reddish in color.

A culture from amniotic fluid added to slanted agar developed into a pure culture of a spirillum.

No guinea pigs inoculated from this case.

Cow 192.—Cow aborted January 23, 1918. Due March 18. Calved 1914 and 1917. Fetus received, frozen, at laboratory. Placenta retained. Length 91 cm., weight 45 pounds. The only noteworthy features of this case are as follows:

The stomachs contained much very viscid light brownish fluid, suspending pieces of meconium up to 7 cm. long, and some hairs. Colon and rectum distended with very dark, tenacious meconium.

Lungs inflated with exception of the left ventral lobe.

Films from surface of mucosa of fourth stomach and rectum negative as regards bacteria.

Cultures from the various organs gave the following result. Two liver, two spleen, and two kidney tubes remained free from growth. Three tubes containing lung tissue and two containing meconium developed into pure cultures of a spirillum. Both tubes containing stomach contents developed heavy growths with gas formation. (Evidently the fetus had breathed and swallowed at birth.)

Two guinea pigs inoculated, one with lung tissue, the other with meconium, were kept 5 weeks, then chloroformed. Lesions and spleen cultures negative.

Cow 213.—Cow calved in 1914, 1915, and 1916. Aborted March 18, 1918. Due June 9. Placenta discharged soon after.

Male fetus, 53 cm. long, hairless. Pelvis unusually narrow; nature of deformity not investigated. Muscular and subcutaneous tissue slightly edematous. Abdomen contains some blood-stained fluid.

Stomachs not overdistended. Rumen contains a thick, tomato sauce-like fluid, viscid, holding in suspension whitish flakes 2 to 3 mm. in diameter, consisting of squamous epithelium (small cell masses from inner surface of amnion). Films show a few spirilla of three to four windings. Fourth stomach contains a still homogeneous, very viscid, slightly amber-colored, transparent fluid, which had to be cut with scissors on account of viscidity, in order to get some for cultures. Rectum distended with small cylindrical masses of dry meconium packed side by side.

Liver of a coarsely mottled yellowish appearance. Spleen several times normal size, flabby. Only one kidney present, this with unusually broad cortex.

Thorax half full of blood-stained fluid. Lungs not inflated. Slight interlobular edema.

Placenta covered with foreign matter and emitting a strong fecal odor. Cotyledons in the main of normal appearance. Some are grayish to whitish. The outstanding feature of the chorion is the presence of slightly elevated patches of an irregular nodular surface usually 3 to 4 cm. in diameter. The nodules are very firm, not readily crushed or pulled away from underlying tissue. Subchorionic tissue edematous. Films from different cotyledons and patches show several varieties of bacteria including spirilla of 1½ to 4 windings. These are more numerous than the other miscellaneous bacteria. Of the cultures prepared two kidney, two spleen, and one liver tube remain sterile. Pure cultures of spirilla are obtained from two lung and two fourth stomach tubes.

Three guinea pigs inoculated respectively with suspensions of lung tissue, contents of fourth stomach, and meconium were chloroformed after 6 weeks and found normal. Of the spleen cultures two tubes contain spore formers, the rest remain sterile.

Microscopic examination of fixed tissues of fetus presents nothing noteworthy. Sections from various regions of placenta show marked edema of chorion and a zone of cell infiltration and cell necrosis between chorion and amnion. The epithelium of chorion has disappeared and the bared margin is densely infiltrated with cells in localized areas. The cells are evidently necrosed, for the nuclei are pyknotic and of bizarre shapes. Some smaller vessels of chorion are nearly closed by lamellated thrombi. Bacteria not detected in these sections. Fibrils of fibrin abundant in tissue spaces and vessels and mask or simulate the presence of bacteria. On the denuded surface of chorion some thick bacilli, and seen only once, a dense mass of spirilla attached to a projecting shred of tissue. The villi of cotyledons not appreciably altered, but most of epithelium has disappeared.

Cow 246.—This cow was purchased September 1917 and aborted in November. This fetus was not obtained. She aborted again during the night of May 27, 1918. Fetus obtained early next morning and refrigerated until May 30.

Fetus still within unbroken membranes. About 20 cm. long. A thick, chocolate-colored fluid within amnion. Skin of fetus easily drawn away exposing partially macerated muscular tissue. Organs in same condition, the liver resembling thick cottage cheese in consistency.

Tubes were inoculated only from liver and lungs. In these pure cultures of spirilla developed.

Two guinea pigs inoculated with a suspension of lungs and liver mixed were chloroformed after 6 weeks. Lesions and spleen cultures negative.

Cow 251.—This cow aborted June 10, 1918. Due October 21. The fetus was found in the manure drop in the morning. Placenta retained.

Fetus female, hairless, 44 cm. long, weight 2,510 gm. Some coils of small intestine hanging out of opening about umbilicus from which blood-tinted fluid escapes. Universal blood-tinted edema of subcutis, of the musculature, and of perirenal space. Liver large, tissue discolored, partly macerated. Stomachs

contain a small quantity of a very turbid yellowish fluid containing whitish particles, composed of squamous cell masses. Large intestines contracted, empty. Thorax half full of blood-stained fluid. Air tubes free from aspirated matter.

Of the tubes inoculated pure cultures of spirilla developed from contents of fourth stomach, small intestine, and lungs. Lung tubes also contained heavy growths in condensation water.

Two guinea pigs were inoculated with suspension of lung tissue and contents of fourth stomach respectively. Chloroformed after 6 weeks, they were found normal and spleen cultures remained sterile.

Cow 256.—Cow purchased in 1914. Calved normally in 1915, 1916, and 1917. Aborted June 17, 1918. Due October 23. Placenta retained.

Fetus, male, length 47 cm., weight 3,925 gm. Hairless. General slight subcutaneous edema, blood-tinted. Large bleb on back containing 95 cc. of blood-stained fluid. 240 cc. of bloody fluid in abdomen and about 50 cc. in pleural sacs. Stomachs contain a thick turbid yellowish fluid. Spirilla detected in films (Fig. 5). Nothing noteworthy about remaining viscera.

Tubes inoculated with bits of tissue and fluids yielded the following results. Two tubes with stomach contents developed mixed cultures of spirilla and other bacteria. Two spleen tubes developed into pure cultures of spirilla. The same is true of two kidney, one lung, and two liver tubes. One other lung tube had a very heavy growth.

Two guinea pigs inoculated, one with stomach contents, the other with lung tissue, were kept 5½ weeks, then chloroformed. They were normal and the spleen cultures remained free from growth.

Sections of fixed and hardened tissues show the presence of numerous short spirilla in four different regions of small intestines. They were limited to the contents.

Cow 258.—Cow purchased August, 1917. Calved in same year. Aborted July 1, 1918. Due September 24. Placenta retained.

Fetus hairless, male, length 57 cm., weight 22 pounds. Abdomen prominent. Testicles swollen, edematous. Subcutaneous edema very slight. In abdomen about 500 cc. of a turbid light reddish fluid, depositing on staining a white sediment made up of leucocytes. Covering liver and adjacent organs is a heavy elastic whitish pseudomembrane, spongy, 1 to 2 mm. thick on liver, and easily stripped off. It contains many cells, appearing as mononuclears in films. Stomachs contain a very viscid fluid, suspending yellowish brown flakes and masses of squamous cells. Liver rather large, yellowish, friable.

Small amount of blood-tinted fluid in pleural sacs. Scattering light yellowish foci in lungs, 2 to 3 mm. in diameter, air-containing. The rest airless. Pericardial cavity contains a turbid, nearly colorless fluid. Epicardium covered with a thin, whitish roughened exudate, giving the heart a furry appearance. Films show spirilla in contents of fourth stomach and rectum.

Tubes of agar inoculated with bits of organs and with fluids from stomachs, etc., gave the following results. Two tubes from fourth stomach and one from

lungs heavily overgrown. Two liver, one lung, two spleen, two kidney, and two meconium tubes develop into pure cultures of spirilla.

Four guinea pigs inoculated, two with meconium and two with lung tissue, were kept 6 weeks and then chloroformed. All were normal and spleen cultures remained free from growth.

In sections of the various organs no noteworthy changes. Spirilla not detected.

Cow 263.—Cow purchased in September, 1915. Calved in 1916 and 1917. Aborted July 30, 1918. Due November 18.

Fetus 51 cm. long, hairless except on lips and chin. Cord remains about 12 cm. long, swollen, edematous. A piece of meconium 5 cm. long protrudes from rectum.

Abdomen contains blood-tinted fluid. Liver slightly softened. Under capsule of kidneys a thin layer of a thick bloody fluid.

Mouth contains a little yellowish, flaky material. Stomachs normally distended with a viscid fluid suspending soft yellowish granules and whitish flakes representing cellular excrescenses of amnion. Films from contents show some short and long spirilla.

Thorax partly filled with a bloody fluid. Trachea contains fluid like that in stomachs.

Cultures from different organs give the following results. Two spleen and two kidney tubes remain sterile. Two stomach, two liver, three lung, and two meconium tubes develop into pure cultures of spirilla.

Three guinea pigs inoculated respectively with contents of fourth stomach, meconium, and lung tissue kept 6 weeks were free from lesions when chloroformed. Cultures from spleens negative.

Cow 267.—Cow purchased July, 1916. Calved 1916 and 1917. Aborted August 12, 1918. Due November 23. Placenta retained.

Fetus, male, hairless, 59 cm. long, weight 6,618 gm. Marked enlargement of abdomen. General subcutaneous edema causing swollen appearance of limbs. Testicles swollen, edematous.

Abdomen contains 650 to 700 cc. of a turbid reddish fluid. White, elastic pseudomembranes cover liver almost entirely and extend over omentum and mesenteries. Easily pulled away.

Stomachs contain a thick, turbid, not very viscid, yellowish fluid. Films show presence of spirilla. Large intestine well filled with meconium. Spirilla present.

Kidneys contain about a dozen cysts, 2 or 3 mm. in diameter, at base of medulla. Spleen with pulp rather soft.

Pleural sacs contain about 50 to 75 cc. of a deeply blood-stained fluid. Minute shreds of whitish exudate sprinkled over all surfaces of the lungs. The air tubes contain a yellowish mucoid fluid like that in stomachs. Spirilla present (Fig. 6).

Cultures of various tissues show the following results. Two lung, two kidney, two spleen, two liver, two fourth stomach, and two meconium tubes develop into pure cultures of spirilla.

Three guinea pigs inoculated respectively with contents of fourth stomach, lung tissue, and meconium and kept over 5 weeks remained well. When they were chloroformed no lesions were found and spleen cultures remained sterile.

EXPLANATION OF PLATES.

All films are stained in alkaline methylene blue.

PLATE 81.

FIG. 1. Section of small intestine, Fetus 256. The figure includes only the contents made up of desquamated cells and three spirilla in focus. Many others, not in view, are present in the cell mass. Eosin-methylene blue. × 1,000.

FIG. 2. Film from contents of trachea of Fetus 267, containing large numbers of long spirilla, brought out by prolonged staining (48 hours). The material consists of regurgitated and aspirated stomach contents. × 1,000.

FIG. 3. Fresh agar culture of spirilla from Fetus 179. Eleventh transfer. × 1,000.

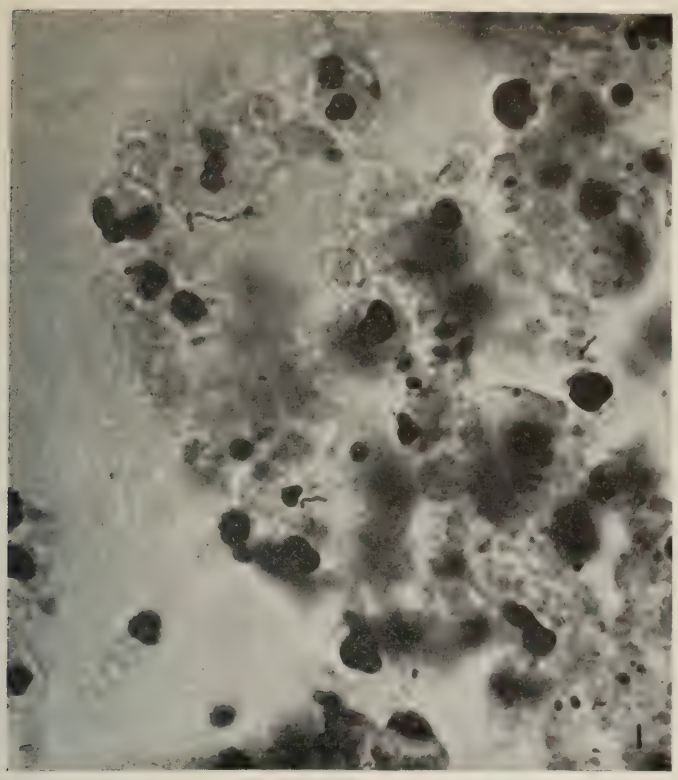
FIG. 4. Old agar culture of spirilla from Fetus 149. Fourteenth transfer. The spirilla are long and beset with deeply stained coccus-like bodies. × 1,000.

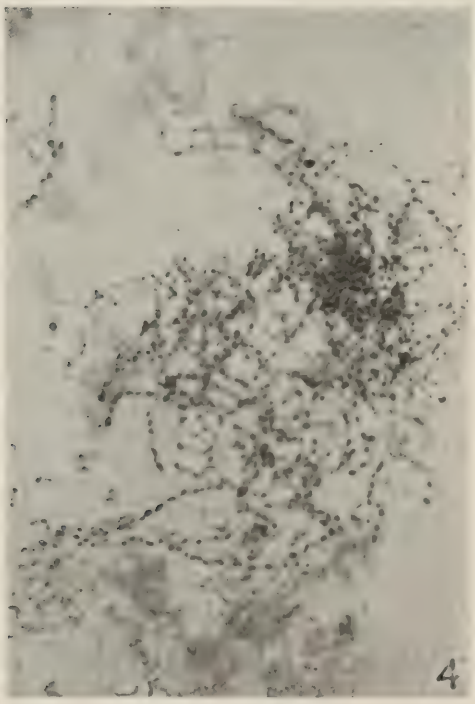
PLATE 82.

FIG. 5. Stained film of contents of rumen, Fetus 256, four spirilla and one degenerated cell in view. × 1,000.

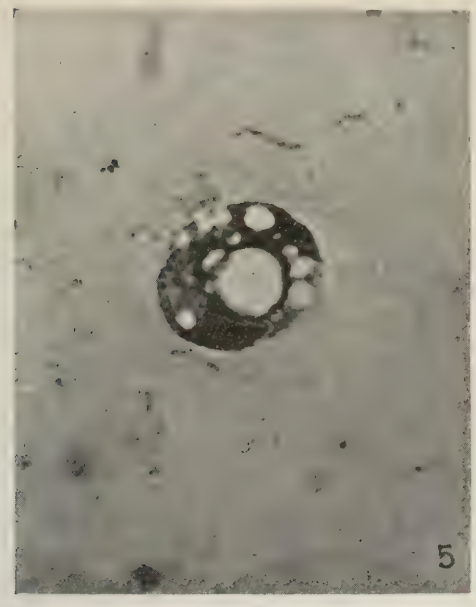
FIG. 6. Two spirilla in film from air tubes of Fetus 267. × 1,000.

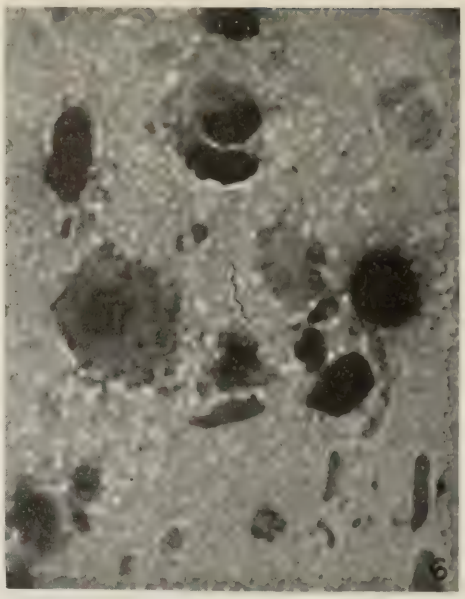
FIG. 7. Transverse section of chorion of Fetus 90, showing the endothelial cells of a blood vessel packed with bacteria which in thin spots have been identified as short spirilla. Cells within lumen of vessel are densely packed with them. Eosin-methylene blue. × 1,000.

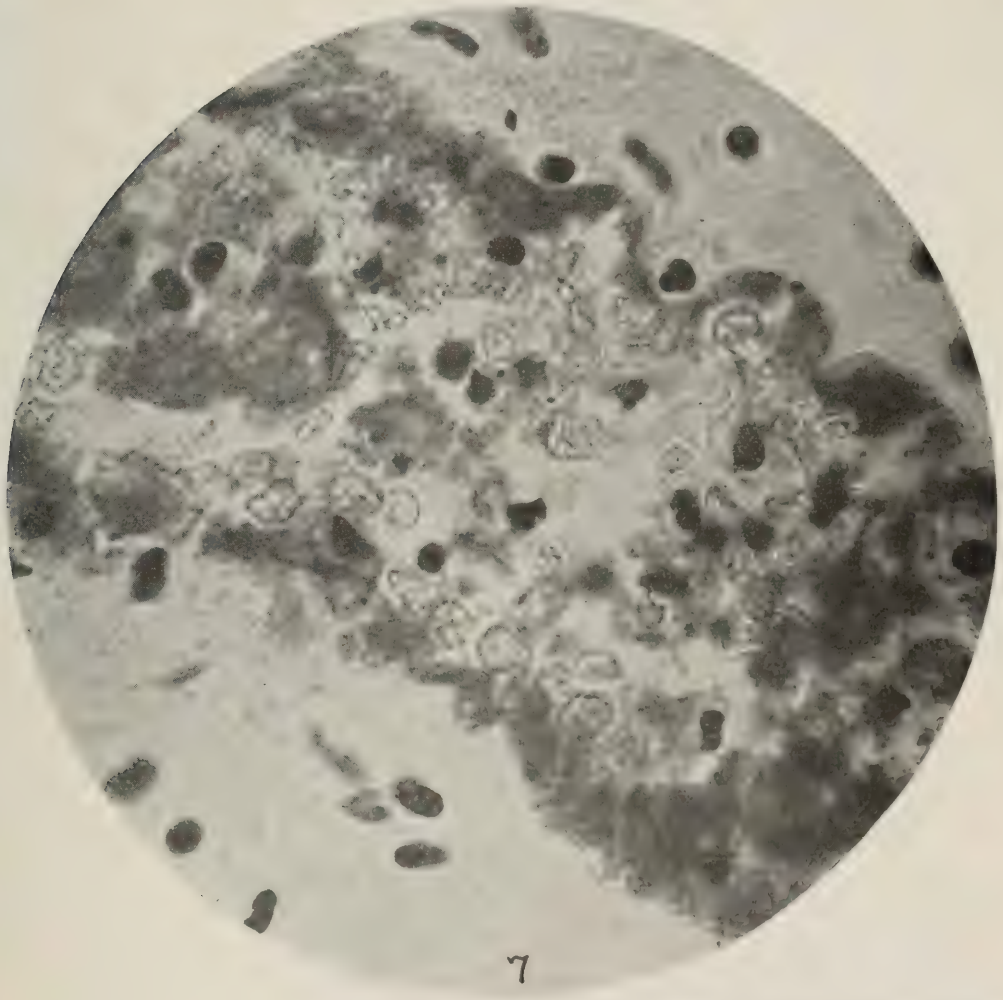




(Smith: Spirilla in infectious abortion of cattle.)







(Smith: Spirilla in infectious abortion of cattle.)

[Reprinted from The Journal of Experimental Medicine, December 1, 1918, Vol. xxviii, No. 6, pp. 721–733.]

STUDIES IN BOVINE MASTITIS.

III. Infection of the Udder with Micrococci and Other Microorganisms.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, August 30, 1918.)

INTRODUCTION.

Many of the earlier investigators noted the presence of micrococci in certain types of udder inflammation. Lucet[1] cultivated gelatin-liquefying, Gram-staining micrococci from seven cases. Guillebeau[2] in studying 85 cases of mastitis found that in 52, micrococci were responsible for more or less severe inflammations. He divided them into two broad groups—the staphylococci which liquefied gelatin and the galactococci which failed to do so. The staphylococci were more frequently found than the galactococci. Steiger[3] reported micrococci associated with udder inflammation in sixteen cases. Like Guillebeau, he classified them as staphylococci and galactococci. Both classes of cocci were described as having a diameter of 1 micron and staining by Gram's method. They also coagulated milk. The staphylococci produced a grayish white, yellow, or golden brown pigment on potato. The liquefaction of gelatin was constant. The galactococci failed to liquefy gelatin but usually produced pigment on potato. Steiger's observations on the pathogenicity of micrococci for cows are interesting. He described eleven cases in cows and one in a goat which cleared up spontaneously within periods varying from 3 days to 3 weeks. On the other hand, his protocols show that streptococci produce a much more severe type of disease. Of the ten cows infected with streptococci, eight suffered from severe mastitis which became chronic.

Savage[4] appears to have been the first to describe in detail the biological characters of the micrococci associated with mammitis. Of the 31 instances which he reported five were associated with staphylococci. He states that the proof

---

[1] Lucet, *Rec. méd. vét.*, 1889, vi, series 7, 423.

[2] Guillebeau, *Landswirtsch. Jahrb. Schweiz.*, 1890, iv.

[3] Steiger, P., *Centr. Bakteriol., Ite Abt., Orig.*, 1904, xxxv, 326, 467, 574.

[4] Savage, W. G., *Rep. Med. Off. Local Gov. Bd. 1907–08*, xxxvii, 425.

of the causal relation of staphylococci to mastitis is unsatisfactory, especially since staphylococci are frequently found in the normal udder. Savage, too, noted the apparent mildness of the disease they produced.

Micrococci Isolated from the Udder.

Of 81 instances of mastitis studied, twenty-four were associated with micrococci. As a rule, the processes which micrococci excite are mild, but in a few instances there has been a serious, acute involvement which became chronic.

The following record is typical of one of the more severe types of infection.

Cow 60.—Dec. 10, 1916. Gave birth to a calf. Sept. 17, 1917. Mastitis developed in the left hind quarter.

Sept. 18. The left hind quarter was swollen, exceedingly firm, and tender. Milk could only be withdrawn with difficulty. Milking was accomplished with considerable pain at first but the discomfort subsided as the pressure was relieved. The milk was yellowish white and thickened with many large flocculi. On standing, many of the larger particles coalesced, forming a large viscid mass on the bottom of the bottle and an excess of fat surmounting a watery liquid. Blood agar plate cultures prepared from the milk revealed 2,172,000 colonies per cc. The surface colonies at the end of 24 hours were 1.5 mm. in diameter, round, and raised. At the end of 48 hours they were larger and had developed a typical orange pigment. The deep colonies were small, round, or ovoid, and non-hemolytic.

Sept. 19. The quarter was still swollen, firm, and tender. The milk was yellow and flocculent.

Cells per cc.	16,000,000
Cocci " "	220,000

The other quarters appeared normal and the milk from them was free from flocculi. Cell counts were well within normal limits. Plate cultures failed to reveal chromogenic micrococci.

Sept. 23.

Cells per cc.	26,000,000
Cocci " "	560,000

From Sept. 23 to 28 the inflammation subsided rapidly. The quarter became more flaccid; a few irregular firm nodules were observed about the milk cystern. The milk was normal in color but still contained a few fine flakes.

Cells per cc.	10,400,000
Cocci " "	1,100

Dec. 2. The quarter appeared normal. The milk was free from flocculi.

Cells per cc	760,000
Cocci " "	1,500

Frequently one observes mild cases associated with only a slight inflammation of the udder. The infected quarter may be slightly enlarged, a trifle firm, and secrete flocculent milk for a day or two. The micrococci usually reach a maximum number when the inflammation is at its height. The number decreases rapidly after the crisis is reached and frequently they disappear from the milk.

Micrococcic infections are not always mild. In a number of instances they have been observed in cases of considerable severity.

Cow 80 developed a severe inflammation of the right hind quarter on September 20, 1917. The quarter was at first enlarged, hot, and painful. The acute inflammation subsided but the quadrant atrophied. The secretion was much reduced and consisted of purulent milk. 6 months later the general condition of the right hind quarter remained unchanged.

Cow 81 calved September 30, 1916. Mastitis developed in the right hind quarter on November 20. After the acute inflammation had subsided a more chronic one continued. The quarter decreased in size, became very firm, and secreted only a few cubic centimeters of flocculent milk. The micrococci were found in the secretion from the affected quarter 5 months later.

In addition, chromogenic micrococci have been isolated from three cases of abscess of the udder. The abscesses were situated in the subcutis and tended to break down and discharge externally. Reparative processes soon set in and the condition cleared up without treatment. Usually they occurred with greatest frequency in cows that came into the herd a short time before. In all probability the infection is spread from animal to animal during the processes of grooming and washing before milking.

Cultural Characters of Micrococci Isolated from Inflamed Udders.

It seemed advisable to cultivate the micrococci in the same media employed in the studies of streptococci. Tubes containing 13 cc. of 1 per cent solutions of the carbohydrates and other substances were used to test the fermentative action of the strains (Table I).

TABLE I.

Morphological and Biological Characters of the Micrococci Isolated from Inflamed Udders.

No. of culture.	Grouping.	Gram's stain.	Chromogenesis on agar.	Milk.	Production of acid in.								Liquefaction of gelatin.
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.	
					per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
C.60	Clumps.	+	Orange.	Firmly coagulated.	3.1	3.0	3.1	2.9	0.9	0.8	3.2	0.8	+
M.W.	"	+	Yellowish white.	" "	3.2	2.9	3.3	2.5	1.6	1.5	2.6	1.1	+
M.4	"	+	Orange.	" "	3.0	2.9	2.5	2.4	0.8	1.0	2.8	1.0	+
M.9	"	+	"	" "	3.0	3.0	2.6	2.3	0.7	1.0	2.2	0.8	+
M.27	"	+	"	" "	3.2	2.8	2.9	2.8	0.7	0.7	2.7	0.6	+
M.32	"	+	Grayish white; translucent.	Coagulated on boiling.	3.5	3.2	2.9	2.8	1.1	0.8	2.1	0.7	+
M.39	"	+	Orange.	Firmly coagulated.	4.1	3.4	3.6	3.2	0.7	0.6	3.4	0.6	+
A.5	"	+	Yellowish white.	" "	3.2	2.8	2.7	2.8	0.9	0.9	3.0	1.1	+
A.8	"	+	Orange.	" "	3.2	2.4	2.4	2.7	0.8	0.9	2.9	0.9	+
A.19	"	+	"	" "	4.0	2.8	3.0	3.8	0.6	0.6	2.6	3.6	+
M.Z.	"	+	White.	Coagulated on boiling.	2.7	2.9	2.9	2.7	0.9	0.9	1.8	0.7	+
M.M.	Clumps.	+	Yellowish white.	Partially coagulated.	3.5	3.1	3.2	1.9	0.0	0.0	2.5	0.0	+
M.1	"	+	Orange.	Firmly "	3.6	3.0	3.0	3.0	0.0	0.0	1.9	0.0	+
M.15	"	+	White.	" "	3.5	3.2	3.4	2.2	0.0	0.0	1.5	0.0	+
M.20	"	+	"	" "	3.9	3.8	3.8	3.5	0.0	0.0	1.6	0.0	+
M.68	"	+	"	" "	3.7	2.9	3.1	2.5	0.0	0.0	2.5	0.0	+
M.70	"	+	Yellowish white.	" "	4.0	3.2	3.3	2.1	0.0	0.1	3.1	0.0	+
M.72	"	+	White.	" "	3.5	3.6	3.5	2.1	0.0	0.0	3.6	0.0	+
M.83	"	+	Orange.	" "	3.1	2.7	2.6	2.5	0.1	0.0	1.7	0.0	+

M.N.	Clumps.	+	White.	Firmly coagulated.	3.4	3.3	3.2	2.1	0.2	0.1	0.2	0.1	+
M.2	"	+	"	Partially "	3.8	3.5	4.0	3.5	0.0	0.1	0.0	0.1	+
M.19	Clumps.	+	Yellowish white.	Firmly coagulated.	3.6	3.2	3.2	2.9	0.1	0.0	2.5	1.0	+
M.49	Clumps.	+	White.	Firmly coagulated.	3.1	2.9	2.7	1.8	1.0	0.1	1.9	0.1	+
M.O.	Pairs.	+	White.	Firmly coagulated.	5.7	3.4	4.0	4.1	2.3	2.1	3.2	2.1	−
M.V.	"	+	Grayish white.	Acid.	2.6	2.1	2.4	2.4	0.6	0.2	1.8	2.3	−
C.80	Clumps.	+	" " translucent.	Firmly coagulated.	3.8	3.0	3.2	3.3	0.0	3.7	1.8	3.0	−
C.81	"	+	" " "	Coagulated on boiling.	3.2	2.8	3.3	3.4	0.9	0.0	1.4	2.9	−
C.69	"	+	White.	Firmly coagulated.	3.0	2.8	2.6	1.8	0.1	0.0	1.3	0.0	−

The figures given beneath the test substances refer to the actual acid production after 5 days incubation at 38°C. Gelatin tubes were incubated at 22°C. for 14 days. Liquefaction is indicated by the sign +.

It will be seen that the micrococci fall into the same broad groups that were noted by Lucet, Guillebeau, Steiger, and others. They termed those liquefying gelatin as staphylococci and those failing to do so as galactococci. From the table it will be readily observed that the larger group is composed of staphylococci. Savage also described both types of micrococci. Further comparison between the cultural characters of the micrococci isolated by the earlier workers and those indicated in the table is impossible. Earlier descriptions were based upon the character of the growth on potato and in gelatin, milk, and bouillon. Savage, however, studied the action of twenty-two strains, isolated from five cases of mastitis, in broth containing maltose, lactose, glycerol, and mannite, as well as in milk. All his strains fermented maltose and glycerol. All but one produced acidity in lactose. Sixteen fermented mannite. In nineteen instances gelatin was liquefied.

In addition to their action upon gelatin many strains possess other minor differential characteristics. Of the twenty-three strains that liquefy gelatin, eleven produce acid in all the test substances. Eight ferment dextrose, lactose, saccharose, maltose, and mannite. Two other species fail to ferment raffinose, inulin, mannite, and salicin. Cultures M.19 and M.49 differ from all the others. The former produces acid in dextrose, lactose, saccharose, maltose, mannite, and salicin. The latter differs only in its failure to ferment salicin.

The number of non-gelatin-liquefying micrococci is too small to warrant any final grouping.

Evans[5] found micrococci in 58.8 per cent of samples of milk drawn directly from the udder. In describing the fermentative action of the staphylococci, dextrose, lactose, maltose, raffinose, mannite, and glycerol were used as test substances, and for this reason comparisons are difficult. There is a great similarity existing between staphylococci isolated from normal and inflamed udders.

A few strains of staphylococci have been tested for their pathogenicity. 1 cc. of 24 hour bouillon cultures of Culture M.M. injected

[5] Evans, A. C., *J. Infect. Dis.*, 1916, xviii, 437.

into the ear vein of a rabbit produced a fatal septicemia within 24 hours. Cultures C.60 and M.15 produced severe involvement of the kidneys; Culture M.72 caused a slight rise in temperature for a few days following the injection. Strains M.N., M.O., and C.80 failed to produce ill effects.

Mastitis Associated with Bacillus coli and Bacillus lactis aerogenes.

Many have described udder inflammations caused by *B. coli communis*. Guillebeau believed at first that he was dealing with a new species. Later, however, Jensen[6] identified Guillebeau's bacilli as *B. coli*. Guillebeau, Jensen, Steiger, and others pointed out that the colon group is responsible for extremely severe mastitis. Savage observed only milder types of infection produced by this organism. Zwick and Weichel,[7] Gilruth and Macdonald,[8] and others have reported outbreaks of mastitis caused by *B. lactis aerogenes*.

Udder inflammations due to this class of organisms have been relatively infrequent in the material at my disposal. The udders of two animals became invaded with *Bacillus coli*. In another instance *Bacillus lactis aerogenes* produced a severe inflammation of one quarter.

Description of Cases Attributed to Colon Bacilli.

Cow 58.—Calved Mar. 19, 1917. July 18. Mastitis developed in the left hind quarter. The quarter was firm, although not appreciably enlarged. The milk was normal in color but contained many small, white flocculi in suspension.

Cells per cc..... 7,500,000
Organisms per cc..... 790 (pure culture of *B. coli*).

The milk from the other quarters appeared normal.

July 20. The left hind quarter was more flaccid. The milk appeared a trifle watery and contained a few flocculi.

Cells per cc..... 1,800,000
Organisms per cc..... 100

B. coli was not observed in the plates.

The other quarters appeared normal. Plate cultures failed to develop *B. coli*.

---

[6] Jensen, C. O., *Ergebn. allg. Path. u. path. Anat. 1897*, 1899, iv, 830.
[7] Zwick and Weichel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 391.
[8] Gilruth, J. A., and Macdonald, N., *Vet. J.*, 1911, lxvii, 217.

July 22. The milk was again examined. All the samples were normal and failed to contain *B. coli*.

Another cow became infected with *Bacillus coli* in the right hind quarter. The quarter was swollen, firm, hot, and painful. The secretion resembled hemolyzed blood and contained large plugs of viscid grayish red material which coalesced on standing into a solid mass on the bottom of the bottle. The secretion contained 264,000 bacilli per cc. The condition became chronic and the animal was slaughtered.

Infection of the Udder with Bacillus lactis aerogenes.

Cow 68.—Calved July 22, 1917. Mastitis was first observed in the left hind quarter on Nov. 11. The quarter appeared normal except for an irregular firm nodule 5 cm. in diameter situated about the milk cystern. The milk was watery and contained large numbers of grayish white flocculi. Plate cultures revealed 26,000 organisms per cc. The surface colonies were round, raised, grayish white, and slimy in appearance. They measured from 3.5 to 4 mm. in diameter after 48 hours incubation. The deeper colonies were round or ovoid.

This cow was under observation for several months. For the first 2 weeks the nodule about the cystern increased in size until it involved the lower third of the quarter. Later it began to decrease, and finally the quarter became flaccid. The secretion gradually declined until only a few cubic centimeters could be withdrawn. Cell counts were not satisfactory, since the flocculi coalesced after washing and centrifugalization. On July 23 the highest cell count of 8,000,000 was recorded. The plate cultures on that day revealed 20,000 colonies of *B. lactis aerogenes* per cc. of milk. The organisms showed a tendency to diminish. On Dec. 17 only 580 colonies were found in a cubic centimeter of milk. The infection persisted throughout the lactation period. The other quarters were not affected.

The cultural characters of *Bacillus coli* and *Bacillus lactis aerogenes* are given in Table II.

Infection of the Udder with Gram-Staining Rod-Shaped Organisms.

In four cases of more or less severity Gram-positive rods have been the only organisms present. In three instances the only apparent symptoms were a transient thickening of the walls of the cystern, usually producing an irregular nodule 4 or 5 cm. in diameter which soon disappeared. The milk was normal except for a varying number of tiny, viscid flocculi. The condition cleared up readily within a few days.

TABLE II.

Cultural Characters of B. coli and B. lactis aerogenes Isolated from Inflamed Udders.

No. of culture.	Motility.	Indol.	Milk.	Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
C.58	+	+	Firmly coagulated.	Gas 44% H 3 CO_2 1 Acid 3.9%	Gas 50% H 4 CO_2 1 Acid 3.5%	Gas: a bubble. Acid 1.5%	Acid 3.8%	Acid 2.4%	Acid 0.0%	Acid 2.9%	Acid 1.9%
M.P.	+	+	Firmly coagulated.	Gas 50% H 7 CO_2 2 Acid 3.9%	Gas 50% H 3 CO_2 1 Acid 3.0%	Acid 0.0%	Acid 3.6%	Acid 0.0%	Acid 0.0%	Acid 2.9%	Acid 0.0%
C.68	−	−	Firmly coagulated.	Acid 4.2%	Acid 4.1%	Acid 4.2%	Acid 3.6%	Acid 2.5%	Acid 0.1%	Acid 2.8%	Acid 3.2%

Cow 55.—This cow represents a more or less typical example of mild infection. In the preceding period of lactation she had suffered from a severe mammitis of the left hind quarter due to non-hemolytic streptococci. Toward the end of the milking period the condition improved but the streptococci were always present in the milk. The cow gave birth to a calf on Nov. 12, 1917. Plate cultures prepared from the milk failed to contain streptococci. On Dec. 26 she suffered from a mild attack of mastitis in the left hind quarter. The quarter appeared normal except for an irregular firm swelling about the cystern. The milk was watery and contained a few flocculi. Plate cultures prepared from the milk revealed 12,300 tiny colonies per cc. The colonies grew slowly and after 72 hours incubation measured 1 mm. in diameter. The attack lasted a few days. Plate cultures prepared on Dec. 29 showed only 200 colonies per cc. The swelling about the cystern had almost disappeared.

A much more severe infection was met with in Cow 79.

Cow 79.—Calved Oct. 8, 1917.

Dec. 27. The animal slipped and fell while walking on the ice. It was supposed that the udder had been injured by the fall, since mastitis made its appearance in the right hind quarter on Dec. 29.

Jan. 8, 1918. The quarter was greatly enlarged, very firm, and hot. Milking caused considerable pain at first but brought relief after a few streams had been withdrawn. The secretion was a thickened greenish white fluid. The odor was offensive. Only 1 pint of milk could be expressed from the quarter. Horse blood agar plate cultures containing 0.00005 cc. of milk revealed countless organisms. Even after the milk had been diluted to 1:50,000 large numbers appeared on the plates after incubation. It was estimated that 700,000,000 colonies developed from 1 cc. of milk. Attempts to count the cells failed because of the unbreakable masses of sediment after centrifugalization. Stained films of milk sediment revealed many polymorphonuclear leucocytes and round cells, and, in addition, large numbers of small rod-shaped organisms and coccoid forms.

Several colonies were fished from the plates and inoculated into serum bouillon. In addition, tubes of melted agar containing a column of medium 8 or 9 cm. deep were inoculated with diluted milk. From the tiny colonies which developed, serum bouillon tubes were inoculated. Plate cultures were prepared from each series of bouillon cultures. In every instance the only organisms developing in the plates were tiny, translucent surface colonies accompanied by deeper biconvex colonies surrounded by a narrow zone of hemolysis. Morphologically all the organisms studied from both series resembled each other.

The condition became chronic. The swelling gradually subsided and ultimately the quarter became smaller than the others. The milk retained its offensive odor.

Mar. 23. The cow was slaughtered. The udder was sent to the laboratory. The right hind quarter was rounded and very firm. On section the lower portion

was principally composed of firm, glistening connective tissue. Many of the larger lactiferous ducts had been obliterated. The milk cystern was constricted. The mucosa of the cystern was necrotic and stained brownish green in an area 2.5 cm. in diameter. Many of the lobules of the upper portion of the gland had been replaced with connective tissue. Others had atrophied. The epithelium was dry and granular.

Microscopically the lesions differed considerably from those found in streptococcic mastitis. Stained sections prepared from the upper portions of the gland revealed, besides an increase of the interlobular tissue, degenerative changes of the epithelium and invasion of leucocytes into the lumen of the acini. Leucocytes were present in considerable numbers in the interacinar vessels. In tissue from the middle and lower portions of the quarter the processes were much more severe. Frequently many lobules had been replaced with connective tissue enmeshing polymorphonuclear leucocytes and round cells. Local areas of hemorrhage had occurred. The connective tissue was edematous in places. A large proportion of the lactiferous ducts had been obliterated. In several the productive process could still be observed. The lining cells of such a duct were compressed. Connective tissue filaments interlacing leucocytes and round cells had invaded the lumen of the vessel. Many newly formed blood vessels were observed in the stroma.

The epithelium of the milk cystern and larger milk ducts had proliferated. The cells had lost their original polyhedral appearance and had become elongated and flattened and resembled squamous epithelium. Finger-like processes frequently extended into the lumen of the duct or into the surrounding connective tissue. Several isolated nests of squamous epithelium were observed in the connective tissue.

Morphological and Cultural Characters of Four Strains of Rod-Shaped Organisms Isolated from Inflamed Udders.

Culture M.T.—Morphology: Tiny non-motile rods. Many show beaded or granular staining with Giemsa's stain. Coccoid forms are not uncommon. The rods stain well by Gram's method.

Colonies in horse blood agar plate cultures:

Surface colonies: Small, round, raised, grayish. 0.5 mm. in diameter after 48 hours incubation.

Deep colonies: Spherical or ovoid. Fail to produce hemolysis in horse blood agar.

Culture C.55 X.—The same morphological and other characters that are described for Culture M.T.

Culture M.44.—Morphology: Very small rods, apparently non-motile. Coccoid forms were not infrequent. Stained preparations revealed many beaded forms. The organisms stain well by Gram's method.

Colonies on horse blood agar:

Surface colonies: Hemispherical, translucent colonies, rarely attaining 0.5 mm. in diameter after 48 hours incubation.

Deep colonies: Ovoid and biconvex surrounded by a narrow hemolytic zone.

Culture C.79.—This culture resembles Culture M.44 in its morphological characters. The growth in horse blood agar plate cultures is the same.

Additional cultural characters are given in Table III. It was necessary to add a small amount of sterile horse serum to each tube of media before good growth could be obtained.

TABLE III.

Biological Characters of Gram-Staining Rod-Shaped Organisms Associated with Mastitis.

No. of culture.	Growth in bouillon.	Milk.	Production of acid in.								Liquefaction of coagulated serum.	Liquefaction of gelatin.	Hemolysis.
			Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.			
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
C.79	Clear.	Firmly coagulated and partially digested.	1.6	1.1	2.9	2.1	0.6	0.5	0.5	0.3	+	−	+
M.44	Turbid.	" "	2.1	1.4	1.5	2.5	0.3	0.6	0.2	0.0	+	−	+
M.T.	Clear.	Unchanged.	Fails to change the reaction of the media.								−	−	−
C.55X.	Turbid.	"	"			"			"		−	−	−

None of the cultures proved pathogenic when injected into the peritoneal cavity of white mice or guinea pigs.

DISCUSSION AND SUMMARY.

Aside from the streptococci, micrococci have been the next most frequent group of organisms isolated from inflamed udders. They produce various types of disease. Some give rise to only a mild catarrh of the larger milk ducts and cystern, while others produce more or less severe parenchymatous inflammation. On the whole, the prognosis is more favorable with micrococcic infection than with

that associated with streptococci. Cases of considerable severity have, however, been attributed to staphylococci.

Micrococci similar in many respects to those associated with mastitis have been found to occur in the normal udder. This has led Savage to question their true etiological significance. In many instances micrococci may gain access to the udder and produce slight disturbances that are entirely overlooked. Even more severe changes may follow infection. After recovery the organisms still remain in the milk. This was observed in the case of Cow 60 infected with staphylococci. One frequently observes the elimination of streptococci from the udder even after apparent recovery from an attack of streptococcic mastitis. Doubtless streptococci and micrococci observed in these udders would be classed as belonging to the normal flora. Even though micrococci do occur in supposedly normal udders Evans has shown that many are pathogenic for rabbits. The introduction of these organisms into the udders of non-resistant individuals might well give rise to more or less intense inflammation. The multiplication would doubtless be rapid until resistance had been established.

In addition to the micrococci two other groups of rod-shaped organisms have been found associated with udder inflammation. In two instances *Bacillus coli* has been isolated from cases of mastitis and in another *Bacillus lactis aerogenes*. In four, tiny motile Gram-staining microorganisms have been obtained in pure culture. Two of these strains (Nos. C.79 and M.44) have been identified as *Bacillus pyogenes*.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, December 1, 1918,
Vol. xxviii, No. 6, pp. 735–748.]

STUDIES IN BOVINE MASTITIS.

IV. THE SOURCES OF INFECTION IN STREPTOCOCCIC MASTITIS.

BY F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, August 30, 1918.)

INTRODUCTION.

Much has been written concerning the etiology of mastitis. Lucet, Guillebeau, Steiger, Savage, Henderson,[1] and others have all isolated streptococci from inflamed udders. During the past year, of the 85 cases of mastitis which have been examined, 54 have been associated with streptococci. Thirty-five cows were infected with the non-hemolytic type and seventeen with the hemolytic. In two instances both types were present.

Mastitis is endemic in the herd that has supplied most of the material for this investigation. The disease is causing more serious losses every year. During 1916, 64 animals were disposed of because of diseased udders. In 1917 the losses increased to 71. Aside from the actual number of animals slaughtered there have been a considerable number of milder cases which recovered. The milk from these animals is discarded as long as it contains flocculi. In addition, one or more quarters frequently atrophy after severe infection.

Streptococcic infections have occurred throughout the herd with extreme irregularity. Attempts to trace infection from one animal to another have usually resulted in failure. It seemed that there must be some source of infection aside from the clinical cases. Many have pointed out the danger of carrying infection on the hands of the milker. Even assuming that the milker might transmit the virus from a clinical case to other cows it could only account for a small

[1] Henderson, J., *J. Comp. Path. and Therap.*, 1904, xvii, 24.

number of infections, especially as the milkers are always instructed to exercise caution in milking known cases.

Three possible sources of infection aside from clinical cases were pointed out in previous studies. Briefly, they may be enumerated as follows: (1) Streptococci are eliminated in the milk before symptoms appear. (2) Frequently they remain in the udder after the disease has subsided. (3) Streptococci may gain access to the udder and persist for a time and then disappear without causing clinical manifestations of mastitis.

Williams[2] and others have suggested the genital tract as a source of infection and point out that mastitis often follows retention of the placenta, metritis, etc. Hagin[3] in bacteriological examinations of the placentas of twenty cows was able to cultivate streptococci from the uterochorionic space in six instances. It is assumed that during parturition these organisms are washed down with the fluids over the buttocks, udder, and teats. They may thus gain access to the teat canal.

With these points in view the milk and the vaginal secretion of 50 cows in one barn in which mastitis had occurred with considerable frequency during the past year were examined. It was postulated that streptococci isolated either from the udder or from the vagina must possess cultural characters and agglutination affinities in common with those previously found associated with mastitis before they could be considered of etiological significance. The writer[4,5] in previous papers has shown that the streptococci found in udder inflammations produced a uniform acidity in dextrose, lactose, saccharose, and maltose. Many fermented salicin, others failed to do so. Milk was always coagulated. In addition, all the non-hemolytic streptococci were agglutinated by an antiserum produced by the immunization of an animal with one strain. The hemolytic types were likewise agglutinated with their specific serum. To conform to the mastitis type, then, it was assumed that a streptococcus must produce acid in dextrose, lactose, saccharose, and maltose. Fer-

[2] Williams, W. L., *Cornell Vet.*, 1918, viii, 63.

[3] Hagin, W. A., *Ann. Rep. New York State Vet. College, Cornell Univ.*, 1916–17, 140.

[4] Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 149.

[5] Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 253.

mentation of salicin was considered variable. It should coagulate milk and be agglutinated by its group serum (hemolytic or non-hemolytic).

Examination of Milk Drawn Directly from the Udders.

Milk was always drawn directly into a sterile wide mouthed bottle. The cows were brushed, washed, and dried before the sample was drawn. The first few streams were discarded. A sample from each quarter was milked into the same bottle. The milk was iced at once and usually plated within an hour. 1 cc. of milk was added to 9 cc. of sterile 0.9 per cent sodium chloride solution and shaken vigorously. 1 cc. of this mixture was added to 12 cc. of 2 per cent veal infusion agar and poured into a Petri dish containing 1 cc. of defibrinated horse blood and the whole mixed. After 24 hours incubation subcultures were made from colonies resembling streptococci. The various media were inoculated from the subcultures which had been proved to be streptococci. Table I shows the arrangement of the cows in the barn and those harboring streptococci in the udder.

From Table I it will be noted that sixteen cows were eliminating streptococci in the milk. During the observation which extended from March until June, 1918, mastitis developed clinically in Cows 10, 21, and 23. Records of the cows showed that during the lactation periods Cows 7, 8, 10, 23, and 37 suffered from mastitis. They had apparently recovered before the milk examinations were begun. The milk from Cows 6, 13, 20, 22, 25, 26, 41, and 50 had never been rejected because of udder inflammation. It must be recognized that such conditions may have been so mild that they escaped detection.

In Tables II and III the morphological and biological characters of the non-hemolytic and hemolytic streptococci isolated from the udders of these apparently normal cows are given. The figures given in the column beneath the various test substances represent the net amount of acid produced in a 1 per cent solution after 5 days incubation.

Since both the non-hemolytic and hemolytic streptococci isolated from the udder were identical in their cultural characters with those

TABLE I.

Arrangement of the Cows in the Barn and Those Harboring Streptococci in the Udder.

Colonies per cc. of milk.	Per cent of streptococci.	No. of subculture.	No. of cow.	No. of cow.	Colonies per cc. of milk.	Per cent of streptococci.	No. of subculture.
1,600	40 per cent hemolytic.	E.25	50	1	900	None.	
1,370	None.		49	2	1,280	"	
600	"		48	3	1,640	"	
1,250	"		47	4	2,800	"	
60	"		46	5	3,100	"	
230	"		45	6	8,000	10 per cent non-hemolytic.	E.2
60	"		44	7	4,000	50 per cent hemolytic.	E.3 E.4
90	"		43	8	9,600	Pure culture hemolytic.	E.5
20	"		42	9	4,700	None.	
8,580	75 per cent non-hemolytic.	E.23	41	10	5,760	Pure culture hemolytic.	E.26 E.6A E.7
480	None.		40	11	500	Pure culture non-hemolytic.	E.8
30	"		39	12	220	None.	
280	"		38	13	400	Pure culture non-hemolytic.	E.9
9,800	70 per cent non-hemolytic.	E.19	37	14	20	None.	
1,700	None.		36	15	900	"	
140	"		35	16	850	"	
50	"		34	17	780	"	
1,060	95 per cent hemolytic.	E.17	33	18	70	"	
480	None.		32	19	6,100	"	
1,300	"		31	20	930	Pure culture hemolytic.	E.10
600	"		30	21	166,000	Pure culture non-hemolytic.	E.11
50	"		29	22	3,750	95 per cent hemolytic.	E.12
6,850	"		28	23	20,800	25 " " non-hemolytic.	E.13
30	"		27	24	2,200	None.	
1,100	40 per cent hemolytic.	E.15	26	25	16,000	80 per cent hemolytic.	E.24

associated with mastitis, it became necessary to test their agglutinative affinity with their specific group serum. The results of these tests are recorded in Tables IV and V.

TABLE II.

Morphological and Biological Characters of the Non-Hemolytic Streptococci Isolated from the Udders of Cows in Table I.

No. of culture.	Grouping in bouillon.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
E.2	M.C.*	+	Turbid.	Firmly coagulated.	4.1	3.6	3.3	3.8	0.0	0.0	0.0	3.4
E.8	"	+	"	" "	4.1	3.3	3.2	3.1	0.0	0.1	0.0	2.4
E.9	L.C.	+	Clear.	" "	4.5	3.8	3.9	4.2	0.0	0.0	0.1	3.9
E.11	M.C.	+	Turbid.	" "	4.6	3.6	3.7	3.8	0.0	0.0	0.0	3.9
E.13	L.C.	+	"	" "	4.6	3.9	3.8	4.4	0.0	0.1	0.2	2.9
E.19	"	+	"	" "	4.6	3.6	3.5	4.0	0.0	0.0	0.0	3.5
E.23	M.C.	+	"	" "	4.5	3.9	3.6	3.9	0.1	0.1	0.0	3.7
E.24	L.C.	+	"	" "	4.5	4.0	3.9	3.7	0.0	0.1	0.0	1.6

*The length of chains has been indicated as follows: L. C., chains of more than 20 elements; M. C., chains composed of 8 to 20 cocci; S. C., chains of less than 8.

TABLE III.

Morphological and Biological Characters of the Hemolytic Streptococci Isolated from the Udders of Cows in Table I.

No. of culture.	Grouping in bouillon.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
E.3	L.C.	+	Turbid.	Firmly coagulated.	4.2	3.8	3.9	4.2	0.0	0.1	0.1	3.0
E.5	"	+	Clear.	" "	4.5	4.2	3.8	3.7	0.0	0.0	0.0	3.0
E.6	"	+	"	" "	4.7	3.9	3.7	3.7	0.0	0.0	0.0	3.2
E.6A.	"	+	"	" "	4.7	4.1	3.8	3.6	0.0	0.0	0.0	2.4
E.7	"	+	"	" "	4.4	3.7	3.8	3.7	0.0	0.0	0.0	2.4
E.10	M.C.	+	Turbid.	" "	4.6	4.0	3.8	3.9	0.0	0.0	0.0	3.1
E.12	"	+	"	" "	4.9	4.1	4.0	4.4	0.0	0.1	0.0	0.0
E.15	L.C.	+	Clear.	" "	4.0	3.5	3.3	3.3	0.0	0.0	0.0	3.0
E.17	"	+	"	" "	3.7	4.0	3.8	3.8	0.1	0.0	0.0	2.9
E.25	"	+	"	" "	4.4	3.6	4.0	4.0	0.1	0.0	0.1	3.7

TABLE IV.

Agglutination Titer of the Non-Hemolytic Streptococci Isolated from the Udder When Tested with the Serum of a Cow Immunized with a Single Strain of Non-Hemolytic Mastitis Streptococci.

No. of culture.	Dilution of serum.						
	1:100	1:500	1:1,000	1:2,000	1:5,000	1:10,000	1:20,000
Mastitis streptococcus used in immunization.	+++*	+++	+++	+++	+++	+++	++
E.2	+++	++	+	+	?	−	−
E.8	+++	+++	+++	+++	+++	++	+
E.9	+++	+++	++	+	−	−	−
E.11	++	+	+	−	−	−	−
E.13	+++	+++	+++	+++	+++	+++	++
E.19	+++	+++	+++	+++	+++	+	?
E.23	+++	+++	+++	+++	+++	++	+
E.24	+++	+++	++	+	−	−	−

* +++ indicates complete agglutination, ++ considerable clumping without entire clearing of the fluid, + a moderate agglutination, − no clumping. A control tube of bacterial suspension to which serum was not added was prepared and incubated in each series.

TABLE V.

Agglutination Titer of the Hemolytic Streptococci Isolated from the Udder When Tested with the Serum of a Rabbit Immunized with a Single Strain of Hemolytic Mastitis Streptococci.

No. of culture.	Dilution of serum.				
	1:100	1:200	1:500	1:1,000	1:2,000
Mastitis streptococcus used in immunization.	+++	+++	+++	+++	++
E.3	+++	+++	+	−	−
E.4	+++	+++	+++	+++	+
E.5	+++	+++	+++	++	+
E.6	+++	+++	++	+	−
E.6A.	+++	+++	+++	+	−
E.7	+++	+++	+++	++	+
E.10	+++	+++	+++	++	+
E.12	+++	+++	+++	++	+
E.15	+++	+++	+++	+	−
E.17	+++	+++	++	+	−
E.25	+++	+++	+	−	−

In addition to the udder, the genital tract of cows was considered a possible source of infection. Mohler and Traum[6] while investigating contagious abortion made bacteriological examinations of the vaginæ of nine normal cows. A considerable number of species of organisms were identified, among them *Streptococcus pyogenes bovis* and *Streptococcus pyogenes albus*. Detailed descriptions of the characters of each species were not given.

Bacteriological examinations of the vaginæ of the 50 cows previously referred to and others that had recently aborted or passed through normal parturition were made. Sterile swabs were inserted into the vagina for a distance of 6 or 8 cm. Within an hour these swabs were agitated for a few seconds in a tube containing 9 cc. of 0.9 per cent sterile sodium chloride solution. After shaking the suspension briskly for a few seconds three platinum loopfuls of the mixture were inoculated into 12 cc. of melted agar cooled to 45°C. The melted agar was poured into a Petri dish containing 1 cc. of sterile defibrinated horse blood and after mixing thoroughly, incubated for 24 hours at 38°C. Subcultures were made from colonies resembling streptococci.

In all, 34 strains of non-hemolytic streptococci have been isolated from the vaginæ of 64 cows. In no instance have hemolytic streptococci been found.

The morphological and cultural characters of all the streptococci isolated from the vagina of cows are given in Table VI. They have been arranged in groups according to their action upon the various test substances.

As a matter of convenience, grouping on the basis of salicin fermentation has not been carried out, especially as acid production by mastitis streptococci in salicin has not been constant. The largest group, representing seventeen strains, attacks mannite. These streptococci have not been found to be associated with mastitis in any of the material studied. The next largest group of eleven strains fails to attack raffinose, inulin, and mannite, and approaches more nearly the types of streptococci which I have isolated from

[6] Mohler, J. R., and Traum, J., *U. S. Dept. Agric., Bureau Animal Industry, 28th Ann. Rep.*, 1911, 147.

TABLE VI.

Morphological and Biological Characters of the Non-Hemolytic Streptococci Isolated from the Vagina of 64 Cows.

No. of culture.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
V 9	M.C.	+	Clear.	Coagulated on boiling.	2.7	2.5	2.7	2.6	0.0	0.0	1.7	0.0
V.15	"	+	"	" " "	2.1	1.7	2.3	1.8	0.1	0.0	1.9	1.9
V.20	Pairs and S.C.	+	Turbid.	Partially coagulated on boiling.	2.1	2.3	2.3	2.3	0.0	0.0	1.9	2.2
V.26	S.C.	+	Clear.	Coagulated on boiling.	3.4	2.8	3.2	2.9	0.0	0.0	2.7	2.3
V.27	M.C.	+	Turbid.	" " "	3.7	3.5	3.5	3.1	0.0	0.0	2.0	3.1
V.28	"	+	"	" " "	3.4	3.0	3.3	3.1	0.0	0.0	2.3	2.6
V.30	S.C.	+	Clear.	Acid 1.2 per cent.	2.2	2.1	2.1	1.7	0.0	0.0	1.9	0.0
V.33	L.C.	+	Turbid.	Coagulated on boiling.	3.9	3.6	3.8	3.4	0.0	0.0	2.3	3.3
V.34	M.C.	+	Clear.	" " "	3.2	2.8	3.0	3.0	0.0	0.0	2.0	2.8
V.35	L.C.	+	Turbid.	" " "	2.6	2.5	2.9	3.0	0.0	0.0	0.7	1.9
V.36	M.C.	+	"	" " "	2.5	2.7	2.7	2.4	0.0	0.0	1.6	0.0
V.37	"	+	"	" " "	2.8	2.6	2.7	2.6	0.0	0.0	1.6	0.0
V.38	S.C.	+	"	" " "	2.7	2.6	2.7	2.5	0.0	0.0	1.9	0.0
V.39	L.C.	+	"	" " "	1.9	1.9	1.8	1.7	0.1	0.2	0.9	1.6
V.43	"	+	Clear.	Unchanged.	2.1	1.1	1.5	1.1	0.0	0.0	1.9	0.3
V.45	"	+	"	Coagulated on boiling.	2.4	2.3	2.1	1.9	0.1	0.0	1.3	1.5
V.47	M.C.	+	Turbid.	Unchanged.	2.6	1.2	1.4	1.0	0.0	0.0	0.8	0.0
V.13	"	+	"	Firmly coagulated.	4.2	3.4	3.6	3.8	0.0	0.1	0.1	3.6
V.14	L.C.	+	"	" "	3.9	3.4	3.6	3.3	0.0	0.1	0.1	3.5
V.16	"	+	Clear.	Acid 0.7 per cent.	2.1	2.1	1.9	2.2	0.1	0.0	0.0	1.8
V.21	Pairs and S.C.	+	Turbid.	Partially coagulated on boiling.	1.9	1.6	1.8	2.0	0.0	0.0	0.1	2.1
V.23	M.C.	+	"	Partially coagulated on boiling.	2.7	2.1	1.7	1.0	0.0	0.0	0.0	0.0
V.29	"	+	"	Acid 0.9 per cent.	2.8	2.8	2.1	2.4	0.0	0.0	0.0	0.0
V.31	L.C.	+	Clear.	Coagulated on boiling.	3.4	3.5	3.3	3.5	0.0	0.0	0.0	2.5
V.32	M.C.	+	"	Acid 1 per cent.	3.4	3.0	3.3	2.8	0.0	0.0	0.0	2.2
V.41	L.C.	+	"	Unchanged.	1.1	1.1	1.1	1.8	0.3	0.3	0.1	0.2
V.44	M.C.	+	Turbid.	Coagulated on boiling.	3.0	4.1	4.0	3.7	0.0	0.0	0.1	3.3
V.49	L.C.	+	"	Unchanged.	2.4	2.4	2.3	2.4	0.0	0.0	0.0	1.1
V.6	M.C.	+	Clear.	Acid 0.7 per cent.	2.0	1.5	2.0	1.3	0.0	0.8	0.0	2.3
V.18	L.C.	+	"	" 0.8 " "	2.2	2.1	1.9	2.2	0.0	0.6	0.1	1.7

TABLE VI—*Concluded.*

No. of culture.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
V.42	Pairs and S.C.	+	Turbid.	Coagulated on boiling.	3.6	2.8	3.7	3.4	1.3	0.1	2.6	3.2
V.46	L.C.	+	Clear.	Unchanged.	2.3	1.2	1.5	1.8	1.1	0.0	1.0	0.8
V.8	Pairs and S.C.	+	Turbid.	Coagulated on boiling.	5.4	4.2	5.4	5.7	5.3	5.7	0.0	4.9
V.25B.	M.C.	+	Clear.	" " "	2.0	2.6	2.3	1.9	1.8	0.0	0.0	2.3

cases of mastitis. Careful examination of the characters of this group, however, reveals distinct differences in many instances. Strains V. 16, V. 21, V. 25B., and V. 49 are low in acid production in the substances which they ferment; in addition, they fail to coagulate milk or produce only sufficient acidity to clot it after boiling. Culture V.29 corresponds somewhat closely with the non-salicin-fermenting type of mastitis streptococci but fails to coagulate milk. The acidity produced by Culture V. 41 is extremely low. In the main the surface colonies on horse blood agar plates have differed from those produced by mastitis streptococci. Vaginal streptococci as a rule produce very tiny, delicate, raised, almost transparent colonies which rarely reach a diameter of 1 mm. after 48 hours incubation. Exceptions, however, are noted in the case of Strains V. 13, V. 14, V. 29, and V. 31. Here the colonies were larger and a trifle more opaque and flattened. Cultures V. 13, V. 14, V. 31, V. 32, and V. 44 possess many characters in common with the streptococci usually associated with mastitis.

As a further means of identification it was decided to test agglutination affinities of the vaginal strains more nearly approaching the mastitis type. The results are shown in Table VII.

The characters of the other six strains of streptococci recorded in Table VI differ to such an extent from those previously isolated from inflamed udders that it seemed useless to consider them further.

Of the 34 vaginal strains two (Strains V.13 and V.14) possessed morphological and cultural characters as well as agglutination affinities in common with the non-hemolytic streptococci associated with mastitis.

TABLE VII.

Agglutination Titer of the Streptococci Isolated from the Vagina.

No. of culture.	Dilution of serum.						
	1:100	1:500	1:1,000	1:2,000	1:5,000	1:10,000	1:20,000
Mastitis streptococcus used in immunization.	+++	+++	+++	+++	+++	+++	+++
V. 13	+++	+++	+++	+++	++	+	−
V. 14	+++	+++	+++	+++	+	−	−
V. 21	−	−	−	−	−	−	−
V. 29	−	−	−	−	−	−	−
V. 31	−	−	−	−	−	−	−
V. 41	−	−	−	−	−	−	−
V. 44	−	−	−	−	−	−	−
V. 49	−	−	−	−	−	−	−

Transmission of the Virus.

It has been difficult to trace the mode of infection. Table I is indicative that infection may take place directly from animal to animal. Cows 7 and 8 both carried hemolytic streptococci, but Cow 6, a neighbor of Cow 7, was infected with the non-hemolytic type. Cow 9 failed to harbor streptococci and Cow 10, which developed mastitis while under observation, eliminated the hemolytic type. Her neighbor, Cow 11, carried a pure culture of non-hemolytic streptococci. It will be observed that the infections traceable to contact are in the minority. This is especially true of Cows 33, 37, 41, and 50. Two other methods of transfer aside from contamination with vaginal discharges remain: (1) transmission of the specific organisms on the hands of the milker and (2) on the curry-combs, brushes, cloths, etc., used in washing the cows.

The "gang" system of milking is employed. Each man is assigned a cow; after milking he washes his hands with soap and cold water and dries them on a fresh towel. He is then assigned to another

cow. In this way one man milks one, two, or three cows irregularly spaced in each barn. Could it be shown that the milker really transmitted the virus on his hands, the irregular occurrence of infections would be readily explained.

The following experiment was devised to test this point. A milker who was known to soil his hands with milk during the process of milking was assigned to milk a cow (No. 10, Table I) known to be eliminating hemolytic streptococci. Before milking, his hands were scrubbed with soap and water and disinfected for 2 minutes in 1:1,000 solution of mercuric chloride. After again washing in water and drying on a clean towel, sterile salt solution was poured over the palms and backs of the hands and collected in a sterile bottle. Salt solution suspensions were prepared with scrapings from beneath the finger nails. His hands were then dried on a fresh towel. After milking, the palms were again rinsed with salt solution. The washings were collected in a sterile bottle. Material from beneath the finger nails was suspended in sterile salt solution. The milker was then instructed to wash his hands in the usual manner (soap and cold water) and dry them. Final washings with salt solution were then collected and scrapings made from the finger nails. The washings and suspensions were brought to the laboratory and plate cultures prepared at once.

Series A.—Plate cultures from washings of hands before milking failed to contain hemolytic streptococci.

Series AA.—Plates prepared from suspensions of nail scrapings before milking failed to contain hemolytic streptococci.

Series B.—Plates prepared from washings of hands after milking contained hemolytic streptococci.

Series BB.—Plates prepared from suspensions of nail scrapings after milking contained hemolytic streptococci.

Series C.—Plates prepared from washings of hands after milking, washing, and drying hands, contained hemolytic streptococci.

Series CC.—Plates prepared from suspensions of nail scrapings after milking, washing, and drying hands did not contain hemolytic streptococci.

To prove definitely that the streptococci found on the hands of the milker were identical with those in the udder, subcultures were inoculated into various media, incubated, and titrated. They were

tested with their specific group serum. The results are given in Tables VIII and IX.

The experiment seems to show that a careless milker may easily carry the virus from an infected to a non-infected cow. This evidence

TABLE VIII.

Morphological and Biological Characters of the Hemolytic Streptococci Isolated from the Udder and from the Hands of the Milker.

No. of culture.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
E.7*	L.C.	+	Clear.	Firmly coagulated.	5.0	4.2	4.3	4.2	0.1	0.1	0.1	2.3
B.†	"	+	"	" "	5.0	3.9	4.5	4.1	0.1	0.1	0.0	2.2
B.B.‡	"	+	"	" "	4.6	4.0	4.4	3.9	0.0	0.0	0.0	2.2
C.§	"	+	"	" "	4.8	4.7	4.4	4.0	0.1	0.1	0.1	2.4

* Strain isolated from the udder.

† Strain isolated from the hands of the milker after milking.

‡ Strain isolated from beneath the finger nails of the milker after milking.

§ Strain isolated from the hands of the milker after they had been washed with soap and cold water.

TABLE IX.

Agglutination Titer of the Streptococci Described in Table VIII When Tested with the Hemolytic Group Serum.

No. of culture.	Dilution of serum.				
	1:100	1:200	1:500	1:1,000	1:2,000
E.7	+++	+++	+++	++	+
B.	+++	+++	+++	+	+
B.B.	+++	+++	+++	++	+
C.	+++	+++	+++	+	+

explains the extreme irregularity of the occurrence of infections. Washing the hands with soap and cold water will not rid them of the virus. A similar experiment was attempted with the non-hemolytic streptococci. They were recovered even after washing. They are

more difficult to recover from the plates because the deeper colonies fail to reveal the more definite characters of the hemolytic types.

Experimental evidence covering infections transmitted by the utensils, water, and cloths used in washing the cows is not available. It seems that if this were the usual method of transmission three or four neighboring animals would be harboring streptococci of the same type. Such has not been frequent.

DISCUSSION.

Infection of the hind quarters of the udder with streptococci has been more frequent than of the fore quarters. In a number of instances the disease appeared immediately after parturition. This was particularly observed in infections associated with the hemolytic types. The evidence was suggestive of infection of genital origin. Bacteriological examination of the vagina of 64 cows revealed, however, non-hemolytic streptococci identical with those associated with mastitis in only two instances. Hemolytic streptococci have not been isolated from the vagina in a single instance. It must be borne in mind, however, that this may not hold true in other herds. All the animals in this study cannot be regarded as normal individuals. A mucopurulent discharge from the vagina was observed in a considerable number of cows. Many had aborted. Others were suffering from vaginitis or metritis. Retention of the placenta had occurred in several instances.

One point which may be of value to those concerned in the study of public health problems is the number of apparently normal cows actually shedding streptococci in the milk. In previous papers it has been shown that these organisms may gain access to the milk supply. It was not known to what extent this held true, but the milk from sixteen cows among 50 in one barn contained streptococci. Nine were harboring the hemolytic type. While the number of cows eliminating such organisms in the milk may not be so high in the average as in this herd, nevertheless, they must exist to a considerable degree. The number of infected samples from this barn is sufficient to enable one to isolate streptococci from samples of the whole supply. The milk is sold as raw milk. If the bovine

streptococci associated with mastitis and identical with them were capable of causing tonsillitis in persons consuming the milk from this dairy, these conditions would have aroused attention and caused an investigation of the milk supply. Such has not been the case.

It must be remembered that in the herd from which a large proportion of the material was obtained the incidence of mastitis was high. In herds where the incidence is low the probable number of carriers would be proportionately low.

CONCLUSIONS.

1. The principal sources of streptococcic infection, aside from clinical cases, are apparently normal cows which carry the virus in the udder.

2. These carriers may be grouped as follows: (*a*) those that have been infected recently and have not yet developed symptoms; (*b*) those that have suffered from inflammation of the udder and after recovery still harbor streptococci; (*c*) those that have had no clinical history of mastitis. There is some evidence to lead one to regard the latter group as naturally immune.

3. A milker may readily carry streptococci on his hands from an infected to an uninfected cow.

4. The vaginæ of 34 of the 64 cows examined contained non-hemolytic streptococci. Of the 34 strains isolated 32 differed in their cultural characters and agglutination affinities from those associated with mastitis. The other two strains may be regarded as of etiological significance.

5. In no instance have hemolytic streptococci been isolated from the vagina.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, December 1, 1918,
Vol. xxviii, No. 6, pp. 749–757.]

THE SURVIVAL OF THE HOG-CHOLERA VIRUS IN LABORATORY ANIMALS, PARTICULARLY THE RAT.

BY CARL TENBROECK, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, September 25, 1918.)

In beginning an investigation of the filterable virus of hog-cholera one line of work decided upon was to study the effect of the virus on various laboratory animals in the hope that some reaction or local infection might be produced that previously had been overlooked. Although Uhlenhuth and Haendel (1) state that the mouse, rat, guinea pig, rabbit, dog, cat, horse, donkey, sheep, cow, goat, pigeon, chicken, goose, and duck are not susceptible to inoculation with the virus, it was nevertheless hoped that by using some of the less common methods of inoculation and by carefully observing the animals some species other than swine might be made available for work on hog-cholera. The method chosen was to inoculate several animals of a given species in one or more ways, to observe them closely, and after approximately 7 days to determine whether the virus was still present in their bodies by inoculation of susceptible pigs.

HISTORICAL.

Craig (2) has reported work along the same line. He injected large amounts of virus (15 cc.) intravenously into rabbits and 8 days later made a suspension of their organs and tissues, and, after passing it through a Berkefeld filter, injected it into pigs. In several instances the animals came down with hog-cholera, showing that the virus had remained alive for 8 days in the body of the rabbit. Attempts to pass the virus from one rabbit to another were negative.

Sir Stewart Stockman (3) attempted to infect wild rats with hog-cholera by feeding them the intestines of pigs showing lesions of the disease. Two lots of rats were fed daily, one for 12 and the other for 11 days, after which they were killed and as much blood as possible was obtained from the heart. The temperature of the pig inoculated with the blood from the first lot rose to between 104° and 105°F. on the 6th day after the injection and remained there for 3 days, after

which it returned to normal. The animal was killed 16 days after the inoculation and no lesions of hog-cholera were found. The pig inoculated with the blood from the second lot of rats showed no temperature or other signs of illness for 42 days following the injection. The result of the inoculation of the first pig must be looked upon as doubtful, but even though it were negative the conclusion that could be drawn would be that the rats did not have the virus in their blood stream and not "that rats are not, as has been suggested, pathological carriers of swine-fever," for the virus might be carried in some organ in the body and eliminated with the urine or feces and still not be present in the blood.

EXPERIMENTAL.

Experiment 1.—Object: To determine whether after inoculation with hog-cholera virus rabbits will show a febrile or other reaction and to determine whether the virus is destroyed in their bodies in 12 days.

A rabbit weighing 1,038 gm. was inoculated intravenously with 1 cc. of sterile, but unfiltered, serum that was proved, by inoculation into a pig, to contain the virus of hog-cholera. On the same day a rabbit weighing 970 gm. was inoculated intraperitoneally with 1 cc. of the same serum. A third rabbit weighing 1,815 gm. received into the testicle 1.4 cc. of the same virus.

The temperature of these rabbits was normal for the next 10 days and there were no visible signs of illness. The rabbit injected into the testicle showed no swelling or local lesion in this organ.

12 days after inoculation the first two rabbits were killed and their abdominal and thoracic viscera and brains fed to a pig weighing 94 pounds. The temperature of this pig was normal for the next 17 days and later it was shown to be susceptible to hog-cholera virus.

Experiment 2.—Will the virus live in the body of the guinea pig 6 days?

A guinea pig weighing 495 gm. was given an intraperitoneal injection of 5 cc. of virulent virus. The animal immediately showed anaphylactic symptoms and its temperature fell to 35.4°C. Another experiment showed that the serum from a normal pig will produce the same effect in guinea pigs. Several hours later the temperature of this animal was normal and it remained so for the next 6 days. It was then chloroformed and a suspension made of pieces of its liver, kidney, lungs, and heart. The spleen was contaminated and was not included. 5 cc. of this thick suspension were injected intramuscularly into a pig weighing 48 pounds. The animal showed no temperature reaction and gained 12 pounds in the next 14 days. It was then inoculated with a virus of very low virulence and showed no reaction. Later it was proved to be immune by inoculation with an active virus and by exposure. I am inclined to believe that the inoculation with the virus of low virulence gave this pig an immunity, but the fact that it was immune makes the interpretation of the experiment doubtful.

Experiment 3.—Object: To determine whether the virus of hog-cholera can be demonstrated in the bodies of pigeons 7 days after inoculation.

Two pigeons were each given an intravenous injection of 1 cc. of active virus and two others were each given an intracerebral injection of ½ cc. of the same virus. During the next 7 days their temperatures were slightly more irregular than those of two uninoculated pigeons kept with them. All the pigeons appeared well and the inoculated ones were chloroformed in 7 days and suspensions made from their livers, spleens, kidneys, hearts, lungs, brains, and pieces of their breast muscle. These organs and tissues were removed, using aseptic precautions, and the suspension was not filtered but was injected directly into a pig weighing 33 pounds. The animal showed no signs of illness for the next 25 days when it was inoculated with virulent virus to which it succumbed. The experiment shows that intravenous and intracerebral injections of hog-cholera virus into pigeons failed to produce disease and 7 days after inoculation the virus could not be demonstrated in their organs and tissues.

Experiment 4.—Four half grown white rats were inoculated with active, sterile, but not filtered, serum from a pig with acute hog-cholera. Two of the rats were given intraabdominal injections of 1 cc. and the other two intracerebral injections of 0.5 cc. of the serum. One of the latter rats died soon after the injection so it will not be considered further. The rats showed no effects from the inoculation and were chloroformed at the end of 7 days. At autopsy no abnormalities were noted. The lungs, hearts, spleens, kidneys, brains, and portions of the livers and muscles of the thigh were passed through a tissue grinder and suspended in an equal amount of sterile salt solution and infused for 5 hours. At the end of this time 9 cc. were injected intramuscularly into the thigh of a susceptible pig weighing 25 pounds. The temperature of this animal was normal for 3 days after which it went up to 40.3°C. on the 4th, 40.5°C. on the 5th, and 40.9°C. on the 6th day following the inoculation. The temperature then fell for 2 days after which it went to 41.5°C. During this time the pig was very sick. It was killed on the 10th day following the injection and showed moderately congested lymph nodes, hemorrhages under the capsule of the kidney, and small ulcers around the ileocecal valve. Cultures from the liver, spleen, and kidney showed no growth. The bacteria-free serum when injected into a susceptible pig produced hog-cholera. We may conclude from this experiment that virulent hog cholera virus remains in the body of the white rat for at least 7 days.

Experiment 5.—Object: To confirm the results of Experiment 4 and to determine whether both types of injection are effective in keeping the virus in the rat's body.

Four rats weighing between 120 and 144 gm. were selected and two of these were each given an intraperitoneal injection of 1 cc. of the serum of the pig used in Experiment 4. The other two were each given an intracerebral injection of 0.5 cc. of the same serum. 7 days after inoculation the two rats that received intraabdominal injections were chloroformed and their livers, spleens, kidneys, testicles, lungs, hearts, and brains passed through a tissue grinder and a 20 per cent suspension was made in salt solution. 10 cc. of this suspension, which represented 2 gm. of tissue, produced typical acute hog-cholera when injected into

a susceptible pig. After sterilizing the instruments and grinder the two rats which had received the intracerebral inoculation were chloroformed, the same organs and tissues as in the other rats were passed through the grinder, and a 20 per cent suspension was made in salt solution. 10 cc. of this suspension also produced typical acute hog-cholera in a susceptible pig. We may conclude from this experiment that the virus of hog-cholera may be demonstrated 7 days after its injection into white rats, whether the injection is intraabdominal or intracerebral.

Experiment 6.—Object: To determine whether the virus is present in the rat 10 days after either intraperitoneal or intracerebral injection.

Two rats were each given an intraperitoneal injection of 1 cc. of the same virus used in Experiment 5, and two other rats were each given an intracerebral injection of 0.5 cc. of the same virus. They were chloroformed after 10 days and a 10 per cent suspension of their organs and tissues was made according to the method used in Experiment 5. The injection of 10 cc. of both of these suspensions into susceptible pigs produced no effect in the month's time that the animals were under observation. The susceptibility of the pigs was not tested but the other animals in the litter were susceptible, so we may conclude that in this experiment the virus of hog-cholera failed to live in the body of the white rat for 10 days after either intraperitoneal or intracerebral injection.

All the above experiments were made with a single strain of virus that had been obtained from Dr. V. A. Moore. It had been passed from pig to pig by inoculation for many generations and it is conceivable that as a result it had been modified so that it was not so susceptible to destructive action of the rat tissues as would be the natural virus.

Experiment 8.—Object: To determine whether another strain of virus recently obtained from the field would live in the rat for 7 days and also to determine in which of the abdominal organs it can be found.

Two white rats were each given an intraperitoneal injection of 1 cc. of a virus two generations removed from the natural strain; that is, the virus had been passed through two of our swine. The rats were chloroformed 7 days after the injection and with separate sterile forceps the spleens, kidneys, and portions of the livers were removed and transferred to weighed Petri dishes. The weights of the organs having been determined, they were ground with sterile sand and 5 per cent suspensions made in salt solution. After standing at room temperature for 3 hours they were injected into pigs. The data for this part of the experiment and the results are given in Table I.

The experiment indicates that 7 days after an injection into the peritoneal cavity of the white rat the virus of hog-cholera is present

in the spleen, it is not present in the liver, and the results of the experiment in as far as the kidneys are concerned are uncertain. It is probable that the pig injected with the kidney suspension suffered from a mild hog-cholera which made it immune. The experiment also shows that this nearly natural strain of hog-cholera virus will live in the rat for at least 7 days.

The above experiments clearly demonstrate that the virus of hog-cholera will live in the body of the white rat for at least 7 days. Rats are constantly associated with swine and it is possible that they

TABLE I.

Pig.		Intramuscular injection of 5 cc. of 5 per cent suspension of rat.	Result.
No.	Weight.		
	lbs.		
A	28½	Spleen.	Temperature up on 4th day. Killed 16 days after inoculation. Kidney hemorrhagic. Ulcers in colon. Filtered urine and serum produced hog-cholera in other swine.
B	28	Liver.	No effects from inoculation. Exposed to Swine A 9 days after inoculation, as a result contracting acute hog-cholera.
C	31	Kidneys.	Temperature slightly above normal on the 5th day after the inoculation but the animal did not appear sick. Subsequent inoculation with filtered urine from typical case of hog-cholera and exposure to pigs with hog-cholera were negative. Killed. No evidences of an old hog-cholera infection found.

may act as intermediate hosts, so the following experiments were made to determine whether or not they could be infected in a more natural way than by injection.

Experiment 9.—Four white rats were fed muscle from a pig that was killed 10 days after it had been injected with virus and while it was moribund. 8 days after the feeding of this infected material two of the rats were chloroformed and a suspension of their spleens, kidneys, hearts, lungs, and portions of their livers was injected into a pig weighing 30 pounds. The animal was under observation 35 days during which time its temperature and general appearance were normal. At the end of this period it received a test inoculation with hog-cholera virus and after 10 days it died, showing on autopsy typical hog-cholera lesions.

The other two rats were allowed to live a month, during which time they appeared normal and when chloroformed and autopsied no abnormalities were found.

In this experiment we were unable to demonstrate the virus of hog-cholera in the bodies of white rats 8 days after they had been fed virus in the shape of meat from a diseased pig.

Experiment 10.—Object: To repeat the attempt to infect rats by feeding material containing hog-cholera virus and in addition to determine whether the virus could be demonstrated in their urine.

Two rats, one weighing 108 and the other 145 gm., were allowed to fast for several hours and then fed pieces of spleen and kidney from a pig dying of hog-cholera. 2 days later they were transferred to an improvised metabolism cage arranged in such a way that their feces were caught in a funnel and their urine was filtered through into a sterile test-tube. Urine was collected on the morning of the 3rd, 5th, and 6th days after the inoculation and placed at once in a refrigerator at approximately 7+° C. The total amount of urine collected from the two rats was about 7 cc. and as this was contaminated with feces it contained many bacteria, but the amount was so small that it did not seem wise to attempt to filter it. The next day after the major portion of urine was collected the whole amount was injected subcutaneously into a pig weighing 51 pounds. The only result of the inoculation was a large abscess at the site of the inoculation. 13 days after the urine was injected the pig was given an intramuscular injection of hog-cholera virus which produced a typical attack with lesions characteristic of hog-cholera.

The rats fed with the virus were killed after 7 days and a 5 per cent suspension was made of their spleens of which 5 cc. were injected intramuscularly into a pig weighing 36 pounds. The animal showed no effects from the inoculation and 15 days later it was given an intramuscular injection of hog-cholera virus to which it succumbed.

In this experiment the attempt to infect white rats by feeding material containing the hog-cholera virus was unsuccessful, as was the attempt to demonstrate the virus in the urine and feces of these same rats.

Experiment 11.—Object: To determine whether a combined infection of hog-cholera virus and hog-cholera bacilli produces in the rat a condition that differs from that caused by the injection of either of these viruses alone.

We have found that at least one of our cultures of the hog-cholera bacillus will produce in the rat a febrile disease either by feeding or by subcutaneous injection. The bacillus can be found in the spleens of these animals at least 21 days after inoculation.

Six rats were weighed, marked, and allowed to fast for 6 hours and then fed meat on which was sprinkled a 24 hour bouillon culture of Hog-cholera Bacillus XII. The next day the rats were divided into lots of three each and the individuals of one lot were each given an intraabdominal injection of 1 cc. of hog-cholera virus that later was proved to be virulent. In addition three uninoculated rats were each given an intraperitoneal injection of the same virus.

The average weights of the three groups of rats show that the gain in weight of the rats that were fed the hog-cholera bacilli was not so great as that in the rats injected with the virus alone, but no visible illness was caused by the combined infection of virus and bacilli.

7 days after the injection of the virus the rats were killed and their abdominal and thoracic viscera fed to pigs weighing about 40 pounds. The animal given the viscera of the rats fed hog-cholera bacilli and injected with hog-cholera virus showed a slight rise in temperature, beginning the 2nd day after the feeding and continuing for 4 days. During this time it was eating and acting normally and for the next 7 days it seemed to be normal in every way. It was then injected with hog-cholera virus to test its susceptibility and died on the 10th day following the inoculation. On autopsy typical lesions of hog-cholera were found and the hog-cholera bacillus was isolated in pure culture from the liver and spleen.

The pig fed the viscera of the rats injected with the virus alone showed no temperature or other evidences of illness during the next 10 days and during this time it gained 15 pounds. It was inoculated with hog-cholera virus to test its susceptibility and 8 days later was chloroformed when it was very sick. Autopsy showed typical lesions of hog-cholera. Cultures were negative.

The experiment shows that a combined infection of hog-cholera bacilli and virus in the white rat caused no more evidences of disease than did the bacilli alone. The attempts to infect pigs by feeding them the viscera of rats that had been injected with hog-cholera virus were negative.

Experiment 12.—Object: To determine whether the virus could be passed from one rat to another.

Three rats weighing between 102 and 113 gm. were each given an intraperitoneal injection of 1 cc. of a serum that later was proved to be active. 7 days after the inoculation the rats were chloroformed and a 10 per cent suspension was made of their ground livers, spleens, and kidneys. 1 cc. of this suspension was injected intraperitoneally into each of three rats weighing between 95 and 98 gm. Several days later these rats were chloroformed and a 10 per cent suspension was made of their livers, spleens, and kidneys. 5 cc. of this suspension were injected intramuscularly into a pig weighing 31 pounds. This pig was apparently normal for the next 24 days. It was then inoculated with virulent virus to which it promptly succumbed, showing that the virus was not present in the second series of rats.

Effect of the Virus of Hog-Cholera on the Rat.

During these experiments the rats inoculated with the virus have been observed carefully and frequently compared with uninoculated animals kept with them. The inoculated rats do not look sick and they gain in weight the same as the controls. Their temperatures show marked fluctuations but this is true of the normal animals and no differences between the two have been detected. With sera and spleen suspensions from inoculated rats and albumin-free but virus-containing urine from hog-cholera pigs as antigen, precipitin and complement fixation reactions have been negative. In other words, the virus apparently produces no disease in the rats.

DISCUSSION AND CONCLUSIONS.

The attempts to demonstrate the virus of hog-cholera in rabbits 12 days after intravenous and intraabdominal inoculations were unsuccessful. Likewise the attempts to show that the virus might be found in the guinea pig 6 and in the pigeon 7 days after inoculation were negative. It was shown, however, that the virus can be found in the bodies of white rats for at least 7 days after either intraabdominal or intracerebral inoculations. One attempt to demonstrate it after 10 days was negative. From the fact that the rats show no evidence of illness such as loss in weight, pyrexia, or visible pathological changes, and that after either intraabdominal or intracerebral inoculation the virus is only found in the abdominal organs and possibly only in the spleen, it seems likely that it does not multiply but that in the rat tissue, particularly in the spleen, it is not destroyed so rapidly as in the organs of other animals. Careful study of the records fails to show that passing one strain of virus alternately through pigs and rats for three transfers in each species changes the virulence for swine or causes the virus to become virulent for rats.

Attempts to introduce the virus into the body of the rat by feeding virulent material and an attempt to pass the virus from one lot of rats to another were unsuccessful, so that we have evidence from the experiment that the rat does not play a part in the transmission of hog-cholera.

BIBLIOGRAPHY.

1. Uhlenhuth, P., and Haendel, L., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, vi, 337.
2. Craig, R. A., *Purdue Univ. Agric. Exp. Station, 29th Ann. Rep.*, 1916, 67, 68.
3. Stockman, S., Great Britain Board of Agriculture and Fisheries, Appendix to final report of the departmental committee appointed to inquire into swine fever, London, 1915, 11.

[Reprinted from The Journal of Experimental Medicine, December 1, 1918, Vol. xxviii, No. 6, pp. 759–777.]

A STUDY OF PARATYPHOID BACILLI ISOLATED FROM CASES OF HOG-CHOLERA.

By CARL TENBROECK, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, September 25, 1918.)

Many swine infected with hog-cholera undoubtedly find their way into our food supply and it would seem that a thorough knowledge of the bacteriology of the disease is of importance from a practical as well as from a theoretical point of view. In the earlier days of the work on this disease attention was chiefly confined to the identification of the hog-cholera and swine-plague bacilli, while since the discovery that a filterable virus is the cause of the condition the bacteriology has to a considerable extent been poorly done or neglected altogether. These so called secondary invaders probably vary with the locality from which the animals are obtained, and also with the virulence and possibly other factors associated with the virus. Accordingly the findings I have to report are a contribution to the bacteriology of the disease rather than a bacteriological study, for only two strains of virus have been used and the majority of the animals came from a single herd. *Bacillus coli*, *pyocyaneus*, *alkaligenes*, several species of anaerobes, and several species of unidentified aerobes have been isolated, but this report will be confined to a description of the paratyphoid bacilli found, because of their economic importance, in that they may be the cause of disease in man, and because they bring up some interesting points in the classification of this group.

HISTORICAL.

The classification of the paratyphoid group is extremely difficult and confusing, for, until recently, there have been no cultural differences between members of the group that could be relied upon, cross-agglutination reactions are common, and animal inoculations have not been made. Dr. Smith (1) stated that the only reliable means of differentiating the hog-cholera bacillus from closely related forms

was by inoculation into rabbits. The former caused a definite disease after subcutaneous injection, resulting in death in from 7 to 12 days with marked changes in the lymphoid tissue, and in freshly isolated cultures with necroses in the liver. The failure to make rabbit inoculations probably accounts for the failure to differentiate the hog-cholera bacillus from paratyphoid β in spite of the statement of Uhlenhuth, Hübener, Xylander, and Bohtz (2) that such inoculations are of no value in differentiating the two.

Dr. Smith noted that the hog-cholera bacillus grew less readily in bouillon than did typhoid or other paratyphoids. Ford (3) suggested that the hog-cholera bacillus could be differentiated from the other members of the group by its failure to ferment arabinose. Jordan (4) and Krumwiede, Kohn, and Valentine (5) have recently shown that the hog-cholera bacillus cultures which they have studied failed to ferment arabinose and in addition that dulcite was fermented little or not at all, and the latter authors have extended the observations of Weiss and Rice (6) on inosite and they show that paratyphoid β is the only member of the group that ferments this carbohydrate. Jordon and Victorson (7) show that the hog-cholera bacillus differs from paratyphoid β in that it fails to form hydrogen sulfide in peptone agar containing lead acetate, whereas the latter and *B. enteritidis* promptly cause a blackening of the medium. A reaction that is somewhat similar is noted by Krumwiede, Kohn, and Valentine (5). They find that in a serum water containing 0.1 per cent dextrose and 1 per cent of Andrade indicator paratyphoid α and the hog-cholera bacillus fail to reduce the fuchsin while paratyphoid β and *enteritidis* give a colorless or at most a faintly pink coagulum at the end of 24 hours incubation.

By means of the agglutination reaction Uhlenhuth, Hübener, Xylander, and Bohtz (2), Savage (8), Jordan (4), and Krumwiede, Kohn, and Valentine (5) agree that paratyphoid α and β and *enteritidis* can be differentiated from one another, but the first authors state that paratyphoid β and hog-cholera bacillus cannot be differentiated by this reaction, while the others say that they can, especially when absorption tests are made.

When one examines the literature of the paratyphoid bacteria, it is found that most workers have considered all members of this group to be hog-cholera bacilli, no matter whether they came from normal or diseased swine. Grabert (9) is often cited as having isolated hog-cholera bacilli from normal swine but the only carbohydrates he used were dextrose, lactose, and saccharose, and in immunizing rabbits two animals survived the injection of 0.01 cc. of a bouillon culture intravenously, which goes a great way towards showing that he did not have typical hog-cholera bacilli. Seiffert (10) compared three strains of what he calls hog-cholera bacilli with paratyphoid β and found that they were the same. It should be noticed, however, that he made no tests for pathogenicity and two of the strains formed acid and gas from arabinose, while the other was not tested, and all of them formed hydrogen sulfide. Not one of the tests used by Uhlenhuth, Hübener, Xylander, and Bohtz would differentiate

the hog-cholera bacilli from other paratyphoids, and as they did not make rabbit inoculations it is impossible to say whether they were working with the true hog-cholera bacillus or with another member of the group. Krumwiede, Kohn, and Valentine (5) seem to think that the hog-cholera bacilli are the only paratyphoids found in swine, for, after showing that paratyphoid β and the hog-cholera bacillus can be separated both culturally and serologically, they conclude that the former is found only in man.

EXPERIMENTAL.

As the cultures to be described came from swine infected with the virus of hog-cholera it seemed wise to repeat the newer cultural tests for the hog-cholera bacillus. Six strains, all of which had been studied by Dr. Smith and pronounced true hog-cholera bacilli, were used and it was found that all six failed to ferment arabinose in 10 days and all but one failed to ferment dulcite in the same time. This one culture which did ferment dulcite failed to do so after it had been passed through a pig, showing how unstable this property is. All the cultures failed to produce a brown color in peptone-lead acetate agar and all gave a decidedly pink color in Krumwiede's dextrose-Andrade-serum water.

The cultures to be described were isolated during the course of some experimental work on hog-cholera in which two strains of virus were used. One of the strains came from Dr. V. A. Moore, while the other was obtained from a spontaneous outbreak in New Jersey. During the work thirty-eight pigs were used, weighing between 20 and 45 pounds. The majority of the animals were raised on the Institute farm where their parents had been isolated from other swine for 3 years. Thirty-three of the pigs were infected by exposure or by the inoculation of bacteria-free material containing the virus, while the remaining five were spontaneous cases obtained from nearby places. Most of the animals were killed in from 7 to 12 days after their temperature began to rise, while some were allowed to die. All showed characteristic lesions of hog-cholera and the sera of many of them were used to infect other pigs.

Cultures were made as a routine from the liver, spleen, and kidney, after searing the surface of the organs, by digging out bits of tissue with flamed forceps and transferring these to agar slants and often-

TABLE I.

Source of Cultures.

Culture No.	From.		History of swine inoculated.			Disposed of.
	Swine No.*	Organ.	Date.	Weight.	Material.	
			1917	*lbs.*		
Swine-typhus I	149	Liver; spleen; kidney.	June 29	18	Fed viscera from spontaneous case of cholera.	July 7. Died. Autopsy 12 hrs. post mortem but animal had been kept cold. Few hemorrhages under capsule of kidney. Fibrinous exudate in cecum. Hemorrhagic pneumonia.
Swine-typhus II	152	Liver; spleen; kidney; lymph node.	July 20		Exposed to spontaneous case.	Aug. 14. Died. Autopsy 5 hrs. post mortem. Typical lesions of hog-cholera.
Swine-typhus III	151	Liver.	Aug. 1	47	Exposed to Swine 152.	Aug. 30. Moribund. Chloroformed and autopsied at once. Typical lesions of hog-cholera.
Swine *enteritidis* I	161	Liver; spleen.	" 21	40	" " fresh spontaneous case of cholera.	Sept. 19. Chronic hog-cholera. Killed. Autopsy shows healing lesions of cholera.
Swine-typhus IV	192	Liver; spleen; kidney.	Dec. 12	21	Inoculated with organ emulsion of rats that had been inoculated with virus 7 days before.	Dec. 19. Sick. Killed. Typical lesions of cholera.
			1918			
Swine typhus V	194	Liver.	Jan. 29	25	Inoculated with filtered emulsion of liver, spleen, and kidney of virus pig.	Feb. 8. Moribund. Chloroformed. Typical lesions of hog-cholera.

* The numbers used belong to a series that was begun many years ago so that they in no way indicate the number of animals used.

times to fermentation tubes containing bouillon. Where growth occurred pure cultures were obtained and from one or more organs of six of the thirty-eight swine bacteria belonging to the paratyphoid group were isolated.

The numbers given to these cultures and a condensed history of the animals from which they were obtained are given in Table I. It is worthy of note that four of the animals were exposed to spontaneous cases of hog-cholera while the other two were purchased from a neighboring farm before inoculation. The animals to which the first four were exposed did not show paratyphoid bacteria in their organs but they must be looked upon as a possible source of the infection. Of a large number of our own animals inoculated with bacteria-free virus not one has shown paratyphoid bacilli in its organs, indicating that they do not carry these organisms in their gastrointestinal tracts.

Cultural Characteristics.

All six cultures resemble one another in that they are motile, Gram-negative rods of about the size of the typhoid bacillus. They grow readily on the ordinary media. On agar they form translucent bluish colonies, while in bouillon they cause a turbidity of about the same density as that caused by the typhoid bacillus. They do not liquefy gelatin or produce indol. They all form acid and gas in fermented bouillon containing 1 per cent dextrose or mannite, while they fail to attack lactose or saccharose. In Durham tubes containing fermented bouillon plus 1 per cent of Andrade indicator and 1 per cent xylose, dulcite, or arabinose they form acid and gas in 24 hours from all three carbohydrates, while neither acid nor gas is produced in salicin bouillon in 10 days. In lead acetate-peptone agar they all produce a brownish color in 24 hours, indicating the formation of hydrogen sulfide. In the medium of Krumwiede made of sterile horse serum one part, sterile distilled water four parts, containing 1 per cent of Andrade indicator and 0.1 per cent dextrose, all six cultures produce in 18 hours a practically colorless coagulum containing gas bubbles. In litmus milk they all give a distinctly alkaline reaction in from 3 to 6 days.

By comparing the cultural reactions of these bacteria with those given for the hog-cholera bacillus it will be seen that there are distinct differences, and from the findings of Jordan and Krumwiede already referred to they would have to be classed as paratyphoid β or *enteritidis*. These two may be differentiated by the fact that the former ferments inosite, but the one test made with the swine cultures was unsatisfactory and no more of the carbohydrate could be obtained.

Pathogenicity.

When injected into the subcutaneous tissues of the rabbit in large numbers these cultures cause a local infection from which the animals recover. The tests are summarized in Table II where it will be seen that 0.1 cc. of a 24 hour bouillon culture failed to kill any of the rabbits.

TABLE II.

Virulence Tested.

Rabbit.		Subcutaneous injection of 0.1 cc. of a 24 hr. bouillon culture of strain.	
No.	Weight.	Culture No.	Effect.
	gm.		
1	2,476	Swine-typhus I	Slight loss in weight with open ulcer at site of inoculation. Recovery.
2	1,572	" I	Slight pyrexia, loss in weight, and local lesion. Recovery.
3	2,505	" II	Slight pyrexia, marked loss in weight, and local lesions. Chloroformed 1 month after inoculation. No lesions found; organs sterile.
4	2,740	" II	Pyrexia, marked loss in weight, and large local lesion. Recovery.
5	2,325	" III	Dead in 6 days. Rabbit septicemia.
6	2,168	" III	Pyrexia, loss in weight, and local lesion. Recovery.
7	2,609	Swine *enteritidis* I	" " " " " " " "
8	2,137	" " I	" " " " " " " "
9	2,500	Swine-typhus IV	Loss in weight and local lesion. Chloroformed 19 days after inoculation. No lesions found and large pieces of liver and spleen were sterile.
10	2,226	" IV	Pyrexia, loss in weight, and local lesion. Recovery.
11	1,167	" V	Amount injected 0.5 cc. Rise in temperature, loss in weight, and large abscess at site of inoculation. Recovery.

In some cases the local lesions were quite extensive and it is probable that had injections been made into the peritoneal cavity death would have resulted. Such injections did not seem necessary as the object was to differentiate these cultures from the hog-cholera bacillus, for, as has been emphasized above, the latter organism always kills the rabbit when injections of 0.1 cc., or even less, of a 24 hour bouillon culture are made into the subcutis. It should also be noted that two of the animals were killed, one 19 and the other 30 days after the injection and that no lesions were found and cultures made by transferring large bits of liver and spleen to bouillon remained sterile, indicating that there had been no general invasion of the body of the animals.

Four mice were fed a bouillon culture of one of these strains (Swine-typhus III). 6 days later one was killed, and though it was apparently well, cultures from the liver and spleen showed a paratyphoid bacillus that was probably the organism ingested. The remaining three animals were not visibly affected by the feeding.

White rats were inoculated with 0.1 cc. of 24 hour bouillon cultures of four of these strains. They showed a slight loss in weight and a local lesion, but they had apparently recovered at the end of 22 days, when they were killed and cultures made from their livers and spleens. Organisms of the paratyphoid group were obtained from two of these rats.

A pig weighing 37 pounds was fed 50 cc. of a 24 hour bouillon culture of Swine-typhus III mixed with ground food, all of which was eaten. The animal showed no effect from the feeding either in its temperature, its appetite, or the consistency of its feces for the next 8 days. It was then inoculated with virulent hog-cholera virus and killed 9 days later when it was very sick. Autopsy showed the typical lesions of hog-cholera. Cultures made from bits of the liver, spleen, and kidney were sterile, showing either that the bacillus was not present in the digestive tract or, if present, it did not have the power to invade the body as does the hog-cholera bacillus when it is fed to a pig and the animal later is inoculated with virus.

The results of the animal inoculations show that the six cultures are not nearly so pathogenic for rabbits as are the hog-cholera bacilli. My experience with paratyphoid β is limited but it seems that these

swine cultures produced larger local lesions than the former does, but this may be due to the fact that the swine cultures have been isolated more recently than the human strains worked with.

Serological Tests.

In preparing animals for immune sera the rabbits used in testing the virulence of the different strains of swine cultures were utilized. After they had recovered from the subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture they were given an intravenous injection of 0.01 cc. of a bouillon culture of the same age. 10 days after the last injection they were bled. This method of treatment gave sera of high agglutination titer but it is possible that such sera differ from those produced by the injection of large numbers of bacteria killed by heat. Uhlenhuth and Hübener (11) state that they get better agglutinating sera for the paratyphoid group by immunizing with killed than with living cultures. Though the organisms are motile they say that the type of agglutination is not characteristic and that all gradations between fluffy and fine clumps may be observed.

When tested against sera produced by the injection of living cultures it was found that one of the strains was sharply marked off by the fact that it failed to be agglutinated by the sera of animals immune to the others and the serum of a rabbit immune to it did not agglutinate the others. The data substantiating these statements are found in Table III.

The serum of this rabbit immune to Swine *enteritidis* I was then tested on other paratyphoids from animals and man and it was shown that the culture is one of *Bacillus enteritidis*, for its serum agglutinates other *enteritidis* cultures as well as the majority of the animal cultures. These consist of two mouse, four rat, three guinea pig, and one dog typhus and two human *enteritidis* cultures. Absorption tests show that these animal and human strains will absorb the agglutinins for the swine as well as for the other strains that are agglutinated by this serum.

The other five strains, which for convenience have been called swine-typhus, appear to be the same serologically as well as culturally. They are agglutinated to the same titer limit and any one of them will absorb the aggultinins for the others.

TABLE III.

Agglutination of Swine Cultures by Sera of Rabbits Immune to Strains That Differ Serologically.

Culture tested.	Limit of agglutination with serum of rabbit.	
	Immune to Swine-typhus III.	Immune to Swine *enteritidis* I.
Swine-typhus I	51,200	100—*
" II	51,200	100—
" III	51,200	80
Swine *enteritidis* I	1,600	51,200
Swine-typhus IV	51,200	100—
" V	12,800+	320
Hog-cholera Arkansas	1,600	80—
" XII	1,600	160—

* Figures followed by a minus sign indicate that there was no agglutination in this the lowest dilution used.

Relation of Swine-Typhus to Hog-Cholera Bacilli.

Though cultural differences have been found between these cultures and those of the hog-cholera bacillus, it is interesting to compare the two serologically.

The agglutination relationship to various hog-cholera bacillus cultures is shown in Table IV, where it will be seen that there is a certain amount of cross-agglutination but the limit of clumping is much lower than in the control strains, and in addition the sediment, after the tubes have been incubated 2 hours and refrigerated over night, is firm and compact, whereas with the homologous strains it is loose and fluffy.

Absorption tests show that cultures of Swine-typhus I and II will take from the anti-hog-cholera bacillus serum the agglutinins for themselves and Swine-typhus V, although the agglutinins for the hog-cholera bacilli are practically unchanged, and that the hog-cholera bacilli will take from the Anti-swine-typhus III serum the agglutinins for themselves without removing those for Swine-typhus III. These facts are shown in Table V.

Thus we see that while there is a certain relationship, as shown by

TABLE IV.

Agglutination Relationship of Hog-Cholera and Swine-Typhus Bacilli.

Culture tested.	Limit of agglutination with serum of rabbit.	
	Immune to Swine-typhus III.	Immune to hog-cholera bacillus.
Swine-typhus I	51,200	12,800×*
" II	51,200	12,800×
" III	51,200	6,400×
" IV	51,200	6,400×
" V	12,800+	1,600×
Hog-cholera bacillus Nebraska	1,600×	
" " Denver	3,200×	
" " Arkansas	3,200×	25,600+
" " X	400×	
" " XI	1,600×	
" " XII	1,600×	12,800

* × indicates that the sediment was firm and compact, +, the highest dilution tested, clumping very marked.

TABLE V.

Absorption of Agglutinins from Hog-Cholera Bacillus and Swine-Typhus Sera by Swine-Typhus and Hog-Cholera Bacilli Respectively.

Serum.			Cultures tested with highest dilution in which agglutination occurred.				
Rabbit No.	Immune to.	Absorbed with.	Swine-typhus II.	Swine-typhus I.	Swine-typhus V.	Hog-cholera Arkansas.	Hog-cholera XII.
12	Hog-cholera bacillus, Arkansas and Nebraska.	Nothing; *i. e.*, control.	12,800	12,800	1,600	25,600+	12,800
12	" "	Swine-typhus I	200	200−	200−	20,000	5,000+
12	" "	" II	200	200	200−	20,000	20,000
12	" "	Hog-cholera XII				500	200−
2	Swine-typhus I	Nothing; *i. e.*, control.		6,400+	6,400+	6,400+	
2	Swine-typhus I	Hog-cholera Arkansas.		6,400+	6,400+	100	

the cross-agglutination between the hog-cholera bacilli and these swine paratyphoids, they can readily be distinguished by the type of clumps formed and by absorption tests.

Relation to Paratyphoid β from Man.

As these strains as far as they have been studied culturally are identical with what Jordan (4) and Krumwiede, Kohn, and Valentine (5) regard as typical paratyphoid β, it is of interest to compare serologically the cultures from man with those from swine. Four paratyphoid β cultures which had been isolated comparatively recently were obtained from Dr. Krumwiede, and when tested it

TABLE VI.

Agglutination of Paratyphoid β Bacilli by Swine-Typhus Sera.

Culture tested.	Limit of agglutination in serum of.			
	Rabbit 13, immune to Swine-typhus I.		Rabbit 6, immune to Swine-typhus III.	
	Limit of agglutination.	Type of sediment.	Limit of agglutination.	Type of sediment.
Swine-typhus I	25,600	Fluffy.		
" III	25,600	"	51,200	Fluffy.
Paratyphoid β 225	6,400	Compact.	1,600	Compact.
" β 232	12,800	"	1,600	"
" β 234	6,400	"	1,600	"
" β 246	12,800	"	800	"

was found that they did not agglutinate to the titer limit with the serum of rabbits immune to the swine-typhus cultures. This is shown in Table VI. The numbers of the paratyphoid cultures are the ones used by Krumwiede, Kohn, and Valentine (5).

Not only do these human cultures fail to agglutinate to the titer limit of the sera but the clumps when first formed are small and granular and after standing in the refrigerator over night the sediment is compact, resembling that formed by non-motile bacteria.

Rabbits were at once immunized against two of the human paratyphoid strains and the swine cultures tested, with the results given in Table VII. It will be seen that the swine cultures fail to agglu-

TABLE VII.

Agglutination of Swine-Typhus Bacilli by Paratyphoid β Sera.

Culture tested.	Limit of agglutination in serum of.			
	Rabbit 14, immune to Paratyphoid β 225.		Rabbit 15, immune to Paratyphoid β 246.	
	Limit of agglutination.	Type of sediment.	Limit of agglutination.	Type of sediment.
Paratyphoid β 225	25,600	Fluffy.	25,600	Fluffy.
" β 246	25,600	"	25,600	"
Swine-typhus I	6,400	Compact.	25,600	Compact.
" II	6,400	"	25,600	"
" III	6,400	"	25,600	"
" IV	6,400	"	25,600	"
" V	3,200	"	6,400	"

TABLE VIII.

Absorption of Agglutinins from Swine-Typhus and Paratyphoid β Sera by Paratyphoid β and Swine-Typhus Bacilli Respectively.

Serum.			Cultures tested with highest dilution in which agglutination occurred.			
Rabbit No.	Immune to.	Absorbed with.	Swine-typhus I.	Swine-typhus III.	Paratyphoid β 232.	Paratyphoid β 246.
13	Swine-typhus I	Nothing; *i. e.*, control.	25,600	25,600	12,800	12,800
13	" I	Paratyphoid β 232	25,600	25,600	200—	200—
13	" I	Paratyphoid β 246	25,600	25,600	200—	200—
			Swine-typhus I.	Swine-typhus III.	Paratyphoid β 225.	Paratyphoid β 246.
14	Paratyphoid β 225	Nothing; *i. e.*, control.	6,400	6,400	25,600	25,600
14	" β 225	Swine-typhus I	200—	200—	12,800	12,800
			Swine-typhus II.	Swine-typhus IV.	Paratyphoid β 225.	Paratyphoid β 246.
15	Paratyphoid β 246	Nothing; *i. e.*, control.	25,600	25,600	25,600	25,600
15	" β 246	Swine-typhus II	200—	200—	6,400	12,800
15	" β 246	" IV	200	200	12,800	12,800

tinate to the titer limit in one serum, while in the other they do agglutinate to the titer limit, but in both sera the sediment is compact, while the sediment formed by the human culture is fluffy, resembling a mass of loose cotton.

Absorption tests, the results of which are given in Table VIII, show that the swine-typhus cultures will not absorb the agglutinins from the human paratyphoid β sera, nor will the paratyphoid β bacilli absorb the agglutinins from the swine-typhus serum.

TABLE IX.

History of Cultures Which Resemble Swine-Typhus Cultures in Their Agglutination.

Culture tested.	History.					Agglutinated by swine-typhus serum, titer 51,200.
	Isolated by.	Date.	Locality.	Source.	Diagnosis.	
Paracolon VI	Dr. N. B. Wherry.	1908	San Francisco, Cal.	Liver of 3 yr. boy.	Status lymphaticus.	25,600
Mouse-typhus I	Dr. Marshal Fabyan.	1911	Mass.	Mouse.	Mouse typhoid.	51,200
Guinea pig-typhus I	Dr. Theobald Smith.	1895	"	Guinea pig.	Pseudotuberculosis.	51,200
Guinea pig-typhus IV	Dr. P. A. Lewis.	1908	"	" "		51,200
Cattle-typhus.	Dr. Mohler.	1904	Washington, D. C. (?).	Cow's brain.	Suspected rabies.	25,600
Pigeon-typhus.	" "	1904	New Jersey.	Heart's blood of pigeon.	Infectious enteritis.	25,600

We have seen that absorption tests show that the hog-cholera bacilli and paratyphoid β differ from these swine-typhus cultures, and the question arises as to whether the last are distinctly different from the paratyphoids that are so widely distributed among various animals. With this question in mind the series of stock cultures collected by Dr. Smith was tested against one of the anti-swine-typhus sera and six cultures were found that agglutinated to the titer limit of the serum and that gave the characteristic fluffy clumps formed by the immunizing strain. It is interesting to note that the cultures from the boy, pigeon, and cow had been sent in as possible

hog-cholera bacilli on account of their unusual virulence for laboratory animals. Culturally they correspond to paratyphoid β or to the swine-typhus cultures and not to the hog-cholera bacillus. The source of these cultures and the date of their isolation are given in Table IX.

It will be seen that the cultures have come from a variety of animals and from widely different localities in the United States. They not only form characteristic clumping in the serum of rabbits immune to the swine-typhus cultures but they absorb the agglutinins for the immunizing culture as is shown in Table X. The

TABLE X.

Absorption of Agglutinins from Swine-Typhus Serum by Paratyphoids from Other Species.

Swine-typhus serum.	
Absorbed with.	Limit of agglutination for immunizing strain.
Nothing; *i. e.*, control	51,200
Paracolon VI	2,500
Mouse-typhus I	2,500
Guinea pig-typhus I	800
" " IV	500
Cow-typhus	400
Pigeon-typhus	3,200

absorption has not been complete in any case but this is probably due to the facts that the serum was of very high titer and not enough bacteria were added. The reduction is, however, great enough to show that an absorption has taken place.

Complement Fixation Experiments.

Another method of studying the relationship of such closely allied groups of organisms is by means of the complement fixation test. Antigens were prepared from two paratyphoid β strains, two swine-typhus strains, and two hog-cholera bacilli cultures as follows:

The growth from one Blake bottle agar slant was suspended in 10 cc. of sterile distilled water and transferred to a sterile bottle

that was tightly closed. The bottles were heated 1 hour in a water bath at 60°C. and then shaken ½ hour, after which they were placed in the refrigerator for 7 days. They were again shaken for ½ hour

TABLE XI.

Complement Fixation Tests Using Swine-Typhus Antigens.

0.05 cc. of serum.		Readings and amounts of antigen.						
		Swine-typhus I.			Swine-typhus III.			
Rabbit No.	Immune to.	0.005 cc.	0.0025 cc.	0.00125 cc.	0.01 cc.	0.005 cc.	0.0025 cc.	0.00125 cc.
13	Swine-typhus I	0	++	++++	0	0	0	++++
16	" III	0	++	C.	0	0	++	++++
15	Paratyphoid β 246	0	++	"	0	0	++	C.
14	" β 225	0	+++	"	0	0	+++	"
12	Hog-cholera Arkansas and Nebraska.	+++	C.	"	C.	C.	C.	"
17	Hog-cholera XII	C.	"	"	"	"	"	"
18	*B. pullorum* III	"	"	"	"	"	"	"
19	Normal.	"	"	"	"	"	"	"

TABLE XII.

Complement Fixation Tests Using Paratyphoid β Antigens.

0.05 cc. of serum.		Readings and amounts of antigen.						
		Paratyphoid β 225.			Paratyphoid β 246.			
Rabbit No.	Immune to.	0.005 cc.	0.0025 cc.	0.00125 cc.	0.01 cc.	0.005 cc.	0.0025 cc.	0.00125 cc.
13	Swine-typhus I	0	0	+++	0	0	0	0
16	" III	0	0	++	0	0	0	0
15	Paratyphoid β 246	0	0	+++	0	0	0	++
14	" β 225	0	0	+++	0	0	0	++
12	Hog-cholera Arkansas and Nebraska.	+++	C.	C.	0	C.	C.	C.
17	Hog-cholera XII	C.	"	"	++++	"	"	"
18	*B. pullorum* III	"	"	"	++	"	"	"
19	Normal.	"	"	"	C.	"	"	"

and placed in the refrigerator for 7 days. At the end of this time 9 cc. of each suspension were added to 1 cc. of 8 per cent sodium chloride solution in a tube and centrifugalized at high speed

for 1 hour. These gave slightly opaque solutions that, on account of their high complement-fixing properties, had to be diluted before they could be used. The hemolytic system used was anti-sheep corpuscle rabbit serum, washed sheep corpuscles, and guinea pig

TABLE XIII.

Complement Fixation Tests Using Hog-Cholera Bacillus Antigens.

0.05 cc. of serum.		Readings and amounts of antigen.							
		Hog-cholera XII.				Hog-cholera Arkansas.			
Rabbit No.	Immune to.	0.01 cc.	0.005 cc.	0.0025 cc.	0.00125 cc.	0.01 cc.	0.005 cc.	0.0025 cc.	0.00125 cc.
13	Swine-typhus I	0	++++	C.	C.	++	C.	C.	C.
16	Swine-typhus III	C.	C.	"	"	+++	"	"	"
15	Paraty-phoid β 246	0	C.	C.	C.	0	++++	C.	C.
14	Paraty-phoid β 225	++++	"	"	"	+++	C.	"	"
12	Hog-cholera Arkansas and Nebraska.	0	+	++++	C.	++	+++	C.	C.
17	Hog-cholera XII	0	0	0	++++	0	++	++++	"
18	*B. pullorum* III	C.	C.	C.	C.	C.	C.	C.	C.
19	Normal.	"	"	"	"	"	"	"	"

complement. The hemolytic amboceptor, complement, and antigens were titrated and all the controls made, but only the actual results of the test are given in Tables XI to XIII. The method used was to decrease the amounts of antigen and use a constant amount of serum. The readings given are those of hemolysis, 0 indicating no

hemolysis or complete fixation of complement, ++ slight, +++ moderate, ++++ almost complete, and C. complete hemolysis.

The results show that the swine-typhus and paratyphoid β antigens act in practically the same manner, causing fixation to occur to about the same degree with sera of animals immune to either group, and causing little or no cross-fixation with sera of rabbits immune to the hog-cholera bacillus. The hog-cholera bacilli antigens are more irregular in their action, for they cause some fixation when used with the sera of rabbits immune to either the swine-typhus or the paratyphoid β cultures, but fixation with hog-cholera sera takes place with higher dilution of the antigens.

In the light of the agglutination tests complement fixation does not give the marked differentiation of the strains that one would expect, but on the contrary, the close interrelation of the members of this group is clearly shown.

Cross-Immunization.

All the above tests show that the five swine-typhus cultures are more closely related to paratyphoid β than they are to the hog-cholera bacillus. It was surprising, therefore, to find that rabbits immune to these swine-typhus cultures were also immune to a virulent hog-cholera bacillus. Animals immune to each of the cultures have been tested and only one, inoculated with Swine-typhus V, died as the result of the injection of hog-cholera bacilli.

The immunity is one of degree only, for while the animals are apparently little affected by the hog-cholera bacilli and live for months, they show upon autopsy a much enlarged spleen, and when large bits of this organ are transferred to bouillon a pure culture of hog-cholera bacillus is obtained. A similar condition in rabbits inoculated with hog-cholera bacilli of low virulence has been described by Dr. Smith (12).

The question naturally arises as to whether paratyphoid β will immunize rabbits to the hog-cholera bacillus, but it will be seen by examining the results given in Table XIV that in two instances the rabbits inoculated with paratyphoid β were not immune, whereas animals treated in the same way with swine-typhus cultures showed

little or no reaction to an inoculation with virulent hog-cholera bacilli. From the results of this experiment it seems that these swine-typhus cultures are more closely related to the hog-cholera bacillus than are the paratyphoid β cultures.

TABLE XIV.

Immunity Tests.

Rabbit No.	Immunization.		Aug. 16, 1918. Subcutaneous injection 1 millionth cc. of 24 hr. bouillon culture of Hog-cholera XII. No. of organisms injected 1,810.	
	Culture used.	Inoculations of bouillon cultures.	Weight.	Result.
		1918	*gm.*	
14	Paratyphoid β 225	July 5. 0.01 cc. subcutaneously. " 22. 0.01 " intravenously.	2,026	Death in 10 days. Typical lesions of hog-cholera bacillus infection.
15	Paratyphoid β 246	" 5. 0.01 " subcutaneously. " 25. 0.01 " intravenously.	1,822	Death in 11 days. Typical findings at autopsy.
13	Swine-typhus I	" 5. 0.01 " subcutaneously. " 25. 0.01 " intravenously.	1,533	Pyrexia, but no loss in weight.
16	Swine-typhus III	" 22. 0.01 " subcutaneously. " 31. 0.01 " intravenously.	1,917	No pyrexia or loss in weight.
20	*B. bronchisepticus.*	" 17. 0.2 " "	1,412	Death in 10 days. Typical lesions.
21	Control.		1,848	Injection one-tenth that given the others. Death in 11 days. Typical lesions.

SUMMARY AND CONCLUSIONS.

During the course of some experimental work on hog-cholera, paratyphoid bacilli were isolated from 16 per cent of the pigs. Culturally these organisms are the same as paratyphoid β isolated from man, while they show several differences from hog-cholera bacilli. In their slight pathogenic effect on rabbits they also differ from the hog-cholera bacillus. In their agglutination in sera produced by the injection of living cultures, one of the cultures, isolated from a chronic case, corresponds to *Bacillus enteritidis*, while the other five are apparently in a class by themselves. They resemble paratyphoid β

more closely than hog-cholera bacilli, but the type of clumps formed and absorption experiments show that they are different from either. Whether these differences are enough to make it necessary to put them into a class by themselves is questionable, but the fact that when injected into rabbits they produce an immunity to the hog-cholera bacillus, while paratyphoid β does not, is additional evidence in favor of such a classification. Complement fixation experiments have been of little value in differentiating the members of this group, but on the contrary show their close relationship. It seems probable that some of the cultures that are described in the literature as hog-cholera bacilli really belong to this group, which would account for much of the confusion that exists in the classification of the interesting, truly pathogenic bacillus that at one time was thought to be the cause of hog-cholera and in the series of animals with which we have worked has not appeared once. Whether the ingestion of pork containing these bacilli would cause disease in man is a question that can only be decided by a more careful bacteriological study of the organisms causing food poisonings and paratyphoid fever.

BIBLIOGRAPHY.

1. Hog cholera: its history, nature, and treatment, *U. S. Dept. Agric., Bureau Animal Industry*, 1889, 69.
2. Uhlenhuth, Hübener, Xylander, and Bohtz, *Arb. k. Gsndhtsamte.*, 1909, xxx, 217.
3. Ford, W. W., *Med. News*, 1905, lxxxvi, 1126.
4. Jordan, E. O., *J. Infect. Dis.*, 1917, xx, 457.
5. Krumwiede, C., Jr., Kohn, L. A., and Valentine, E., *J. Med. Research*, 1918, xxxviii, 89.
6. Weiss, H., and Rice, J. L., *J. Med. Research*, 1916–17, xxxv, 403.
7. Jordan, E. O., and Victorson, R., *J. Infect. Dis.*, 1907, xxi, 554.
8. Savage, W. G., *J. Hyg.*, 1912, xii, 1.
9. Grabert, K., *Z. Infectionskrankh., parasit. Krankh. u. Hyg. Haustiere*, 1917, iii, 218.
10. Seiffert, G., *Z. Hyg. u. Infectionskrankh.*, 1909, lxiii, 273.
11. Uhlenhuth, P., and Hübener, E., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, iii, 1021.
12. Smith, T., *U. S. Dept. Agric., Bureau Animal Industry, 12th and 13th Ann. Rep., 1895–96*, 1897, 161.

INDEX TO AUTHORS.

A

Amoss, Harold L., and **Eberson, Frederick.** An experimental test of Nuzum's antipoliomyelitic serum, 21

— and **Haselbauer, Peter.** The rat and poliomyelitis: an experimental study, 69

— and **Marsh, Penelope.** Standardization of antimeningococcic serum, 203

Avery, O. T. See DERNBY and AVERY, 409

B

Brown, James Howard. A new type of syringe, especially adapted for intravenous injections or the aspiration of blood, 509

— and **Orcutt, Marion L.** A rapid differential method for the isolation of *Bacillus influenzæ*, 515

C

Chesney, Alan M. See CULLEN and CHESNEY, 401

Cullen, Glenn E., and **Chesney, Alan M.** A note on the production of acid by pneumococci, 401

— and **Taylor, Herbert D.** Relative irritant properties of the chlorine group of antiseptics, 183

D

Defandorf, J. See KLIGLER and DEFANDORF, 51

Dernby, Karl Gustav. A study on autolysis of animal tissues, 359

Dernby, Karl Gustav, and **Avery, O. T.** The optimum hydrogen ion concentration for the growth of pneumococcus, 409

Doust Brewster C., and **Lyon, Arthur Bates.** Face masks in infections of the respiratory tract, 423

E

Eberson, Frederick. See AMOSS and EBERSON, 21

G

Gates, Frederick L. A report on antimeningitis vaccination and observations on agglutinins in the blood of chronic meningococcus carriers, 75

—. Restorative effect of salts of magnesium and calcium after lethal doses of sodium oxalate, 9

Githens, T. S., Kleiner, I. S., Meyer, A. L., and **Meltzer, S. J.** A method of producing experimental shock, 215

—, —, —, and —. Influence of mere opening the abdomen, of state of shock, and of subsequent section of sciatic nerve upon the blood flow from the femoral vein in cats, 217

Goto, Kingo. Studies of acidosis. XII. Mineral metabolism in experimental acidosis, 437

H

Haselbauer, Peter. See AMOSS and HASELBAUER, 69

Heidelberger, Michael. See JACOBS, HEIDELBERGER, and ROLF, 277

J

Jacobs, Walter A., Heidelberger, Michael, and **Rolf, Ida P.** On nitro- and aminoaryl arsonic acids, 277

Jones, F. S. Studies in bovine mastitis. II. The relation of hemolytic streptococci to udder infections, 459

—. III. Infection of the udder with micrococci and other microorganisms, 539

—. IV. The sources of infection in streptococcic mastitis, 553

K

Kaliski, David J. See NOGUCHI and KALISKI, 141

Kleiner, I. S. See GITHENS, KLEINER, MEYER, and MELTZER, 215, 217

Kligler, I. J. Modifications of culture media used in the isolation and differentiation of typhoid, dysentery, and allied bacilli, 17

—. Note on cross-agglutination of *Bacillus coli communis* and *Bacillus dysenteriæ* Shiga, 55

— and **Defandorf, J.** The endo medium for the isolation of *Bacillus dysenteriæ* and a double sugar medium for the differentiation of *Bacillus dysenteriæ*, Shiga and Flexner, 51

L

Levene, P. A. The action of nitrous acid on epimeric hexosaminic acids, 249

—. Epimeric hexosaminic acids, 233

— and **López-Suárez, J.** Mucins and mucoids, 255

— and **Taylor, F. A.** Reduction of aldehydes to corresponding alcohols. I. Reduction of heptylic aldehyde (oenanthol), 223

Levene, P. A., and **West, C. J.** Cephalin. V. Hydrocephalin of the egg yolk, 227

Loeb, Jacques. Amphoteric colloids. I. Chemical influence of the hydrogen ion concentration, 303

—. II. Volumetric analysis of ion-protein compounds; the significance of the isoelectric point for the purification of amphoteric colloids, 341

—. The influence of electrolytes upon the osmotic pressure of gelatin solutions, 291

—. The law controlling the quantity of regeneration in the stem of *Bryophyllum calycinum*, 325

López-Suárez, J. See LEVENE and LÓPEZ-SUÁREZ, 255

Lyon, Arthur Bates. See DOUST and LYON, 423

M

MacNider, William deB. The stability of the acid-base equilibrium of the blood in naturally nephropathic animals and the effect on renal function of changes in this equilibrium. I. A study of the acid-base equilibrium of the blood in naturally nephropathic animals and of the functional capacity of the kidney in such animals following an anesthetic, 101

—. II. A study of the efficiency of an alkali to protect the naturally nephropathic kidney against the toxic effect of an anesthetic, 117

Marsh, Penelope. Survival of typhoid bacilli in sour milk, 31

—. See AMOSS and MARSH, 203

Meltzer, S. J. The application of a concentrated solution of magnesium sulfate to scalds and burns, 219
—. See GITHENS, KLEINER, MEYER, and MELTZER, 215, 217
—. See WOLLSTEIN and MELTZER, 129, 133
Meyer, A. L. See GITHENS, KLEINER, MEYER, and MELTZER, 215, 217

N

Noguchi, Hideyo. A comparative study of experimental prophylactic inoculation against *Leptospira icterohæmorrhagiæ*, 143
—. Influence of temperature upon the velocity of the complement fixation reaction in syphilis, 1
— and **Kaliski, David J.** The spirochetal flora of the normal female genitalia, 141

O

Oliver, Jean. See ROUS and OLIVER, 153
Orcutt, Marion L. See BROWN and ORCUTT, 515

R

Rolf, Ida P. See JACOBS, HEIDELBERGER, and ROLF, 277
Rous, Peyton. Urinary siderosis. Hemosiderin granules in the urine as an aid in the diagnosis of pernicious anemia, hemochromatosis, and other diseases causing siderosis of the kidney, 169
— and **Oliver, Jean.** Experimental hemochromatosis, 153

S

Smillie, Ernest W. An improvement in the method of isolating and recovering the bacillus of cattle abortion through guinea pigs, 487
Smith, Theobald. A pleomorphic bacillus from pneumonic lungs of calves simulating actinomyces, 475
—. Spirilla associated with disease of the fetal membranes in cattle (infectious abortion), 519

T

Taylor, F. A. See LEVENE and TAYLOR, 223
Taylor, Herbert D. See CULLEN and TAYLOR, 183
TenBroeck, Carl. A study of paratyphoid bacilli isolated from cases of hog-cholera, 577
—. The survival of the hog-cholera virus in laboratory animals, particularly the rat, 567

U

Uhlenhuth, Eduard. The antagonism between thymus and parathyroid glands, 37
—. Further proof of the existence of a specific tetany-producing substance in the thymus gland, 47

V

Van Slyke, Donald D. Studies of acidosis. XI. The determination of carbon dioxide in carbonates, 433

W

West, C. J. See LEVENE and WEST, 227
Wollstein, Martha. A further study of experimental parotitis, 59
— and **Meltzer, S. J.** Effect of intrabronchial insufflation of solutions of some inorganic salts, 133
— and —. Experimental chemical pneumonia, 129

INDEX TO SUBJECTS.

A

Abdomen:
Opening, influence of, on blood flow from femoral vein in cats (GITHENS, KLEINER, MEYER, and MELTZER) 217

Abortion:
Cattle, bacillus, isolation and recovery through guinea pigs (SMILLIE) 487
Infectious, spirilla associated with (SMITH) 519

Acid(s):
Aminoaryl arsonic (JACOBS, HEIDELBERGER, and ROLF) 277
Arsonic, aminoaryl (JACOBS, HEIDELBERGER, and ROLF) 277
—, nitroaryl (JACOBS, HEIDELBERGER, and ROLF) 277
-base, equilibrium of blood in naturally nephropathic animals (MACNIDER) 101, 117
—, — — — in naturally nephropathic animals, effect on renal function of changes in (MACNIDER) 101, 117
Epimeric hexosaminic (LEVENE) 233
— —, action of nitrous acid on (LEVENE) 249
Nitroaryl arsonic (JACOBS, HEIDELBERGER, and ROLF) 277
Nitrous, action of, on epimeric hexosaminic acids (LEVENE) 249

Acid(s)—*Continued:*
Production, by pneumococci (CULLEN and CHESNEY) 401

Acidosis:
Metabolism, mineral, in experimental (GOTO) 437
Studies (GOTO) 437
— (VAN SLYKE) 433

Actinomyces:
Pleomorphic bacillus from pneumonic lungs simulating (SMITH) 475

Agglutination:
Cross-, of *Bacillus coli communis* and *Bacillus dysenteriæ* Shiga (KLIGLER) 55
—, of *Bacillus dysenteriæ* Shiga and *Bacillus coli communis* (KLIGLER) 55

Agglutinin:
Meningococcus carriers, chronic (GATES) 75

Alcohols:
Aldehydes, reduction of, to corresponding alcohols (LEVENE and TAYLOR) 223

Aldehydes:
Alcohols, reduction from corresponding aldehydes (LEVENE and TAYLOR) 223
Reduction of heptylic aldehyde (oenanthol) (LEVENE and TAYLOR) 223

Alkali:
Kidney, naturally nephropathic, protected against anesthetic by (MACNIDER) 117

Aminoaryl arsonic acid:
See Acid.
Analysis:
Volumetric, of ion-protein compounds (LOEB) 341
Anemia:
Pernicious, diagnosed by hemosiderin granules in urine (ROUS) 169
Anesthetic:
Kidney, naturally nephropathic, functional capacity (MACNIDER) 101
—, — —, protected by alkali against (MACNIDER) 117
Antagonism:
Thymus and parathyroid glands (UHLENHUTH) 37
Antimeningococcic serum:
See Serum, meningitis.
Antipoliomyelitic serum:
See Serum, poliomyelitis.
Antiseptic:
Chlorine, irritant properties (CULLEN and TAYLOR) 183
Arsonic acid:
See acid.
Aspiration:
Blood, syringe (BROWN) 509
Autolysis:
Animal tissues (DERNBY) 359

B

Bacillus:
Cattle abortion, isolation and recovery through guinea pigs (SMILLIE) 487
coli communis, cross-agglutination with *Bacillus dysenteriæ* Shiga (KLIGLER) 55
dysenteriæ. *See* Dysentery.
influenzæ, rapid differential method for isolation (BROWN and ORCUTT) 515
Bacillus—*Continued:*
Paratyphoid. *See* Paratyphoid.
Pleomorphic, from pneumonic lungs (SMITH) 475
typhosus. *See* Typhoid.
Base:
Acid-, equilibrium of blood in naturally nephropathic animals (MACNIDER) 101, 117
—, — — — in naturally nephropathic animals, effect on renal function of changes in (MACNIDER) 101, 117
Blood:
Acid-base equilibrium, in naturally nephropathic animals (MACNIDER) 101, 117
Agglutinins, of chronic meningococcus carriers (GATES) 75
Aspiration, syringe (BROWN) 509
Flow from femoral vein in cats (GITHENS, KLEINER, MEYER, and MELTZER) 217
Bryophyllum:
calycinum, regeneration in stem (LOEB) 325
Burn:
Magnesium sulfate solution on burns (MELTZER) 219

C

Calcium:
Salts, restorative effect after lethal doses of sodium oxalate (GATES) 9
Calycinum:
Bryophyllum, regeneration in stem (LOEB) 325
Carbonates:
Carbon dioxide in (VAN SLYKE) 433
Carbon dioxide:
Determination of, in carbonates (VAN SLYKE) 433

Carrier:
Meningococcus, chronic, agglutinins (GATES) 75
Cattle:
Abortion, bacillus, isolation and recovery through guinea pigs (SMILLIE) 487
—, infectious, spirilla associated with (SMITH) 519
Mastitis, bovine (JONES) 459, 539, 553
Cephalin:
Hydrocephalin of the egg yolk (LEVENE and WEST) 227
Chemical:
Hydrogen ion concentration, chemical influence (LOEB) 303
Pneumonia (WOLLSTEIN and MELTZER) 129
Chlorine:
Antiseptics, irritant properties (CULLEN and TAYLOR) 183
Cholera:
Hog-, paratyphoid bacilli isolated from (TENBROECK) 577
—, virus, survival in animals (TENBROECK) 567
Coli communis:
Bacillus, cross-agglutination with *Bacillus dysenteriæ* Shiga (KLIGLER) 55
Colloids:
Amphoteric (LOEB) 303, 341
—, significance of isoelectric point for purification (LOEB) 341
Complement:
Fixation reaction in syphilis, influence of temperature on velocity (NOGUCHI) 1
Culture:
Media, modifications, for isolation and differentiation of typhoid, dysentery, and allied bacilli (KLIGLER) 17

D

Diagnosis:
Anemia, pernicious, by hemosiderin granules in urine (ROUS) 169
Diseases causing siderosis of kidney, by hemosiderin granules in urine (ROUS) 169
Hemochromatosis, by hemosiderin granules in urine (ROUS) 169
Disease:
Fetal membranes in cattle, spirilla associated with (SMITH) 519
Kidney siderosis, diseases causing, diagnosed by hemosiderin granules in urine (ROUS) 169
Dysenteriæ:
Bacillus. *See* Dysentery.
Dysentery:
Bacillus, cross-agglutination with *Bacillus coli communis* (KLIGLER) 55
—, differentiation, double sugar medium for (KLIGLER and DEFANDORF) 51
—, isolation and differentiation from allied bacilli (KLIGLER) 17
—, —, endo medium for (KLIGLER and DEFANDORF) 51

E

Egg:
Yolk, hydrocephalin (LEVENE and WEST) 227
Electrolytes:
Gelatin solutions, electrolytes on osmotic pressure of (LOEB) 291
Epimeric hexosaminic acid:
See Acid.

Equilibrium:
Acid-base, of blood, in naturally nephropathic animals (MacNider) 101, 117
—, — —, in naturally nephropathic animals, effect on renal function of changes in (MacNider) 101, 117

F

Face:
Masks in infections of respiratory tract (Doust and Lyon) 423

Female:
Genitalia, normal, spirochetal flora of (Noguchi and Kaliski) 141

Femur:
Vein, femoral, blood flow from, in cats (Githens, Kleiner, Meyer, and Meltzer) 217

Fetus:
Membranes, of cattle, disease, spirilla associated with (Smith) 519

Fixation:
Complement, reaction, in syphilis, influence of temperature on velocity (Noguchi) 1

Flora:
Spirochetal, of normal female genitalia (Noguchi and Kaliski) 141

Function:
Kidney, effect of changes in acid-base equilibrium of blood in naturally nephropathic animals (MacNider) 101, 117
—, functional capacity in naturally nephropathic animals after anesthetic (MacNider) 101

G

Gelatin:
Solution, electrolytes upon the osmotic pressure of (Loeb) 291

Genitalia:
Spirochetal flora of normal female (Noguchi and Kaliski) 141

Gland:
Parathyroid, antagonism between thymus and (Uhlenhuth) 37
Thymus, antagonism between parathyroid and (Uhlenhuth) 37
—, specific tetany-producing substance in (Uhlenhuth) 47

Granule:
Hemosiderin, in urine, in diagnosis of diseases causing siderosis of kidney (Rous) 169

Growth:
Pneumococcus, optimum hydrogen ion concentration (Dernby and Avery) 409

H

Hemochromatosis:
(Rous and Oliver) 153
Diagnosis by hemosiderin granules in urine (Rous) 169

Hemolytic:
Streptococci, relation to udder infections (Jones) 459

Hemosiderin:
Granules, in urine, in diagnosis of diseases causing siderosis of kidney (Rous) 169

Hexosaminic acid:
See Acid.

Hog-cholera:
See Cholera.

Hydrocephalin:
Egg yolk (LEVENE and WEST) 227
Hydrogen ion:
Concentration, chemical influence (LOEB) 303
—, optimum, for pneumococcus growth (DERNBY and AVERY) 409

I

Icterohæmorrhagiæ:
Leptospira, prophylactic inoculation (NOGUCHI) 143
Infection:
Abortion, infectious, spirilla associated with (SMITH) 519
Respiratory tract, face masks in (DOUST and LYON) 423
Udder, hemolytic streptococci (JONES) 459
—, micrococci and other microorganisms (JONES) 539
—, sources in streptococcic mastitis (JONES) 553
Influenzæ:
Bacillus, rapid differential method for isolation (BROWN and ORCUTT) 515
Injection:
Intravenous, syringe (BROWN) 509
Inoculation:
Prophylactic. *See* Vaccination.
Inorganic:
Salts, solutions, intrabronchial insufflation (WOLLSTEIN and MELTZER) 133
Insufflation:
Intrabronchial, of solutions of inorganic salts (WOLLSTEIN and MELTZER) 133
Intrabronchial insufflation:
See Insufflation.
Ion-protein:
Compounds, volumetric analysis (LOEB) 341
Irritation:
Chlorine antiseptics, irritant properties (CULLEN and TAYLOR) 183
Isoelectric point:
Amphoteric colloids, significance of isoelectric point for purification (LOEB) 341

K

Kidney:
Nephropathic, naturally, effect of changes in acid-base equilibrium on function (MACNIDER) 101, 117
—, —, functional capacity after anesthetic (MACNIDER) 101
—, —, protected by alkali against anesthetic (MACNIDER) 117
Siderosis, diseases causing, diagnosed by hemosiderin granules in urine (ROUS) 169

L

Leptospira:
icterohæmorrhagiæ, prophylactic inoculation (NOGUCHI) 143
Lues:
See Syphilis.
Lungs:
Pneumonic, pleomorphic bacillus from (SMITH) 475

M

Magnesium:
Salts, restorative effect after lethal doses of sodium oxalate (GATES) 9
Sulfate solution on scalds and burns (MELTZER) 219

Masks:
Face, in infections of respiratory tract (DOUST and LYON) 423
Mastitis:
Bovine (JONES) 459, 539, 553
Streptococcic, sources of infection (JONES) 553
Medium:
Culture, modification in, for isolation and differentiation of typhoid, dysentery, and allied bacilli (KLIGLER) 17
Endo, for isolation of *Bacillus dysenteriæ* (KLIGLER and DEFANDORF) 51
Sugar, double, for differentiation of *Bacillus dysenteriæ*, Shiga and Flexner (KLIGLER and DEFANDORF) 51
Membrane:
Fetal, of cattle, spirilla associated with disease of (SMITH) 519
Meningitis:
Serum, antimeningococcic, standardization (AMOSS and MARSH) 203
Vaccination, antimeningitis (GATES) 75
Meningococcus:
Carriers, chronic, agglutinins (GATES) 75
Metabolism:
Mineral, in experimental acidosis (GOTO) 437
Method:
Bacillus influenzæ, isolation, rapid differential method (BROWN and ORCUTT) 515
Cattle abortion, bacillus, isolation and recovery through guinea pigs (SMILLIE) 487
Method—*Continued:*
Shock, experimental, method of producing (GITHENS, KLEINER, MEYER, and MELTZER) 215
Micrococcus:
Udder infection (JONES) 539
Microorganism:
Udder infection (JONES) 539
Milk:
Typhoid bacilli in sour (MARSH) 31
Mucins:
Mucoids and (LEVENE and LÓPEZ-SUÁREZ) 255
Mucoids:
Mucins and (LEVENE and LÓPEZ-SUÁREZ) 255
Mumps:
See Parotitis.

N

Nephropathy:
Acid-base equilibrium of blood (MACNIDER) 101, 117
Kidney, functional capacity, after anesthetic (MACNIDER) 101
—, protected by alkali against anesthetic (MACNIDER) 117
Nerve:
Section of sciatic, influence upon blood flow from the femoral vein in cats (GITHENS, KLEINER, MEYER, and MELTZER) 217
Nitroaryl arsonic acid:
See Acid.
Nitrous acid:
See Acid.

O

Oenanthol:
Reduction of oenanthol (heptylic aldehyde) (LEVENE and TAYLOR) 223

Osmosis:
Gelatin solutions, electrolytes on (LOEB) 291
Oxalate:
Sodium. *See* Sodium.

P

Parathyroid:
Antagonism between thymus and (UHLENHUTH) 37
Paratyphoid:
Bacilli, isolated from hog-cholera cases (TENBROECK) 577
Parotitis:
(WOLLSTEIN) 59
Pleomorphism:
Bacillus, pleomorphic, from pneumonic lungs (SMITH) 475
Pneumococcus:
Acid production (CULLEN and CHESNEY) 401
Growth, optimum hydrogen ion concentration (DERNBY and AVERY) 409
Pneumonia:
Chemical (WOLLSTEIN and MELTZER) 129
Lungs, pleomorphic bacillus from (SMITH) 475
Poliomyelitis:
Rat, relation to (AMOSS and HASELBAUER) 69
Serum, Nuzum's antipoliomyelitic (AMOSS and EBERSON) 21
Pressure:
Osmotic, of gelatin solutions (LOEB) 291
Prophylaxis:
Inoculation. *See* Vaccination.
Protection:
Alkali, in naturally nephropathic animals, against anesthetic (MACNIDER) 117
Purification:
Amphoteric colloids, significance of isoelectric point (LOEB) 341

R

Rat:
Poliomyelitis, relation of rat (AMOSS and HASELBAUER) 69
Reaction:
Complement fixation, in syphilis, influence of temperature on velocity (NOGUCHI) 1
Regeneration:
Bryophyllum calycinum, stem (LOEB) 325
Renal:
See Kidney.
Respiratory tract:
Infections of, face masks in (DOUST and LYON) 423

S

Salt(s):
Calcium, restorative effect after lethal doses of sodium oxalate (GATES) 9
Inorganic, solutions, intrabronchial insufflation (WOLLSTEIN and MELTZER) 133
Magnesium, restorative effect after lethal doses of sodium oxalate (GATES) 9
Scald:
Magnesium sulfate solution on scalds (MELTZER) 219
Section:
Nerve, sciatic, influence upon blood flow from the femoral vein in cats (GITHENS, KLEINER, MEYER, and MELTZER) 217

Serum:
Meningitis, standardization (Amoss and Marsh) 203
Poliomyelitis, Nuzum's (Amoss and Eberson) 21
Shock:
Experimental, method of producing (Githens, Kleiner, Meyer, and Meltzer) 215
State, influence on blood flow from femoral vein in cats (Githens, Kleiner, Meyer, and Meltzer) 217
Siderosis:
Kidney (Rous) 169
—, diseases causing, diagnosed by hemosiderin granules in urine (Rous) 169
Sodium:
Oxalate, lethal doses of, restorative effect of calcium salts (Gates) 9
—, — — —, restorative effect of magnesium salts (Gates) 9
Solution:
Gelatin, electrolytes upon the osmotic pressure of (Loeb) 291
Magnesium sulfate on burns (Meltzer) 219
— — — scalds (Meltzer) 219
Salts, inorganic, intrabronchial insufflation (Wollstein and Meltzer) 133
Spirillum:
Infectious abortion (Smith) 519
Spirochæta:
Genitalia, normal female, spirochetal flora of (Noguchi and Kaliski) 141
Standardization:
Antimeningococcic serum (Amoss and Marsh) 203
Stem:
Bryophyllum calycinum, regeneration (Loeb) 325
Streptococcus:
Hemolytic, relation to udder infections (Jones) 459
Mastitis, sources of infection (Jones) 553
Substance:
Tetany-producing, specific, in thymus gland (Uhlenhuth) 47
Sugar:
Medium, double, for differentiation of *Bacillus dysenteriæ*, Shiga and Flexner (Kligler and Defandorf) 51
Sulfate:
Magnesium, solution of, on scalds and burns (Meltzer) 219
Survival:
Hog-cholera virus in animals (TenBroeck) 567
Typhoid bacilli in sour milk (Marsh) 31
Syphilis:
Complement fixation reaction, influence of temperature on velocity (Noguchi) 1
Syringe:
Blood aspiration (Brown) 509
Injections, intravenous (Brown) 509

T

Temperature:
Syphilis complement fixation reaction, influence of temperature on velocity (Noguchi) 1
Tetany:
-producing substance, specific in thymus gland (Uhlenhuth) 47
Thymus:
Antagonism between parathyroid and (Uhlenhuth) 73

Thymus—*Continued:*
Gland, antagonism (UHLENHUTH) 37
—, specific tetany-producing substance in (UHLENHUTH) 47
Tissue:
Animal, autolysis of (DERNBY) 359
Toxicity:
Anesthetic, protection of naturally nephropathic kidney by alkali against (MACNIDER) 117
Typhoid:
Bacillus in sour milk (MARSH) 31
—, isolation and differentiation from allied bacilli (KLIGLER) 17
Typhosus:
Bacillus. *See* Typhoid.

U

Udder:
Infection, hemolytic streptococci (JONES) 459
Udder—*Continued:*
Infection, micrococci and other microorganisms (JONES) 539
—, sources in streptococcic mastitis (JONES) 553
Urine:
Hemosiderin granules in, in diagnosis of diseases causing siderosis of kidney (ROUS) 169
Siderosis (ROUS) 169

V

Vaccination:
Leptospira icterohæmorrhagiæ (NOGUCHI) 143
Meningococcus (GATES) 75
Vein:
Blood flow from femoral, in cats (GITHENS, KLEINER, MEYER, and MELTZER) 217
Virus:
Hog-cholera, survival in animals (TENBROECK) 567

R
111
R7
v. 32

Rockefeller Institute, New York
Studies; reprints

Biological
& Medical
Serials

PLEASE DO NOT REMOVE
CARDS OR SLIPS FROM THIS POCKET

UNIVERSITY OF TORONTO LIBRARY